Universidade de Aveiro Secção Autónoma das Ciências da Saúde

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Daniel Filipe Soares Pereira da Cruz

Lifestyle impact on human sperm oxidative balance

Impacto do estilo de vida no equilibrio oxidativo do sémen humano

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Margarida Sâncio da Cruz Fardilha, Professora auxiliar convidada da Secção Autónoma das Ciências da Saúde da Universidade de Aveiro e de Doutor Luís Korrodi-Gregório, investigador de Pós-Doutoramento, Centro de Biologia Celular, Universidade de Aveiro.

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palavras-chave

Espermatozoide, antioxidantes, stress oxidativo, estilo de vida, infertilidade masculina.

resumo

A infertilidade é um problema clínico que afeta cerca de 15% dos casais em idade fértil. Metade dos casos de infertilidade deve-se a fatores masculinos, sendo que uma grande percentagem tem origem idiopática. Nos últimos anos tem-se discutido a influência do stress oxidativo na diminuição da qualidade seminal. O estilo de vida, nomeadamente o consumo de álcool e tabaco têm sido fatores propostos como responsáveis pelo aumento de espécies reativas de oxigénio (ROS), levando a uma alteração do equilíbrio entre os oxidantes e as defesas antioxidantes presentes no organismo, causando stress oxidativo. Níveis aumentados de ROS danificam as biomoléculas – DNA, proteínas ou lípidos – presentes nos espermatozoides, podendo levar à perda da integridade da membrana, à fragmentação de DNA ou até mesmo à morte por apoptose.

Este estudo tem como objetivo avaliar a influência da alteração aguda do estilo de vida no equilíbrio oxidativo dos espermatozoides. Desta forma, foi analisada a capacidade antioxidante dos espermatozoides, bem como a presença de certas proteínas antioxidantes, através de técnicas colorimétricas e de immunoblotting. Foi também avaliado o efeito das ROS através da medição de oxidação proteica. A qualidade seminal foi avaliada através da realização de espermogramas.

Os resultados obtidos indicam que existe uma relação entre as alterações do estilo de vida e a quantidade de antioxidantes no espermatozoide, sendo que a alteração mais marcada envolveu a proteína superóxido dismutase (SOD). Foi também detetado uma variação dos níveis de oxidação proteica, dependente da alteração dos consumos de álcool e nicotina.

Com este trabalho concluiu-se que o equilíbrio oxidativo dos espermatozoides é afetado pelas alterações no estilo de vida, sendo que a alteração deste equilíbrio reflete-se posteriormente na qualidade seminal.

keywords Spermatozoon, antioxidants, oxidative stress, lifestyle, male infertility.

abstract

Infertility is a clinical condition that affects about 15% of reproductive-aged couples worldwide. Half of these cases are due to male factors. A large percentage of male infertility cases are idiopathic, however, in the last years the influence of oxidative stress in decreased semen quality has been discussed. The lifestyle, including consumption of alcohol, tobacco, drugs and the alteration in circadian cycle, has been proposed as responsible for the increase of reactive oxygen species (ROS). This increase leads to an alteration of the balance between oxidants and antioxidant defenses present in the organism, causing oxidative stress. High levels of ROS damage biomolecules – DNA, proteins or lipids – present in sperm cells and may lead to the loss of membrane integrity, DNA fragmentation or even to death by apoptosis.

The aims of the thesis was to evaluate the influence of acute lifestyle changes on oxidative balance of sperm cells. Therefore, we analyzed the antioxidant capacity of the sperm, as well as the presence of certain antioxidant proteins, by colorimetric techniques and immunoblotting. We also evaluated the effect of ROS by measuring the protein oxidation. The seminal quality was evaluated by performing a routine semen analysis.

The results indicate that there is a relationship between the changes in lifestyle and the amount of antioxidants in sperm, and the most reported change involved the protein superoxide dismutase (SOD). It was also demonstrated that the variation in protein oxidation levels is dependent on the consumption of alcohol and nicotine.

In this study it was concluded that oxidative balance of sperm cells is affected by lifestyle changes; in turn, oxidative balance changes is then reflected in semen quality.

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ABREVIATIONS

ABTS	2,2-Azino-Di-(3-Ethylbenzthiazoiine Sulphonate)
AIF	Apoptosis Inducing Factor
APS	Ammonium Persulfate
APU	"*Associação Portuguesa de Urologia*"
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CAT	Catalase
CG	Carbonyl Groups
DNA	Deoxyribonucleic Acid
DNPH	2,4-Dinitrophenylhydrazine
DTNB	5,5'-Dithiobis(2-Nitrobenzoic Acid)
G6PD	Glucose-6-Phosphate-Desidrogenase
GPx	Glutathione Peroxidase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
HOST	Hypo-Osmotic Swelling Test
LB	Loading Buffer
LGB	Lower Gel Buffer
MDA	Malondialdehyde
NAC	N-Acetylcysteine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NOX5	Nicotinamide Adenine Dinucleotide Phosphate Oxidase 5
3-NT	3-Nitrotyrosine

8-OHdG	8-Hidroxy-Deoxyguanosine
OS	Oxidative Stress
PBS	Phosphate Buffered Saline
PHGPx	Phospholipid Hydroperoxide
PUFA	Polyunsaturated Fatty Acid
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
SSRI	Selective Serotonin Reuptake Inhibitor
TAS	Total Antioxidant Status
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline and Tween-20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
TP	Time Point
UGB	Upper Gel Buffer
WHO	World Health Organization
WR	Working Reagent

1. INTRODUCTION

1.1. Spermatozoon

Spermatozoon is the male gamete produced in the testis by spermatogenesis, and its main function is to fertilize the oocyte to form an egg and consequently an embryo (Jonge & Barratt, 2005) (Figure 1).

This cell is divided into two sections: head and tail or flagellum. The cell is completely covered by an unique cell membrane, also called plasmalema (Jonge & Barratt, 2005). The head of human spermatozoon has a flattened and pointed shape and it comprises the nucleus, the acrosome and the plasma membrane. The nuclear DNA is condensed and it is connected to protamines, which replace histones during spermatogenesis. Protamines are positively charged proteins that are responsible for the hypercondensation of spermatozoon nucleus, which favors a higher motility and head penetration into the oocytes. The nucleus is covered by a reduced nuclear envelope, in which the majority of the pore complex was removed during spermiogenesis. The perinuclear theca protects the nucleus, by forming a rigid shell composed of structural proteins stabilized by disulphide bonds (Jonge & Barratt, 2005). The acrosome is a secretory organelle derived from the Golgi complex and it is very similar to a cellular lysosome. This organelle is responsible for storing secretory content for extended periods of time. The acrosome also possesses several hydrolytic enzymes, such as hyaluronidase, neuraminidase and acid phosphatase, which are responsible for digesting the *zona pellucida* of the oocyte.

The tail of the spermatozoon is responsible for the motility, having a single axoneme with 9+2 microtubule arrangement disposed in a crown, corresponding to two central plus nine microtubules pairs. The tail is divided into 4 regions:

(i) connecting piece;
(ii) middle piece;
(iii) principal piece and
(iv) end piece.

The connecting piece has the centrioles and is the place of origin of the outer dense fibers. In the middle piece are localized the mitochondria (organelles responsible for the energy supply for the tail motion), that surround the outer dense fibers. The principal piece is the longest region of the sperm and contains the fibrous sheaths, the outer dense fibers and the axonemal complex. The end piece, with only 5 mm in length, contains axonemal complex and the ends of the outer dense fibers and fibrous sheath (Jonge & Barratt, 2005).

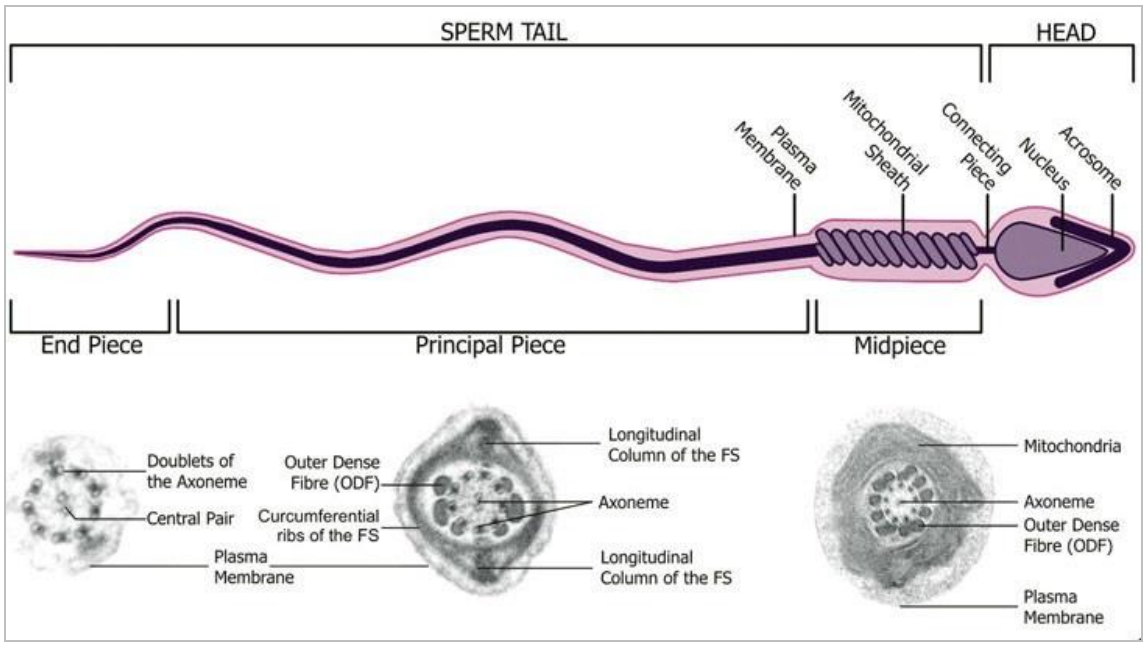

Figure 1 - Schematic representation of the spermatozoon and its components. Spermatozoon is composed by the head and the tail. The tail comprises connecting piece, midpiece, principal piece and end piece. The electron micrographs showing cross-sections of each region highlights the main components of the tail structure: the axoneme; outer dense fibers (ODF); and the mitochondrial sheath (midpiece) and fibrous sheath (FS) (principal piece). The end-piece consists of the axoneme and plasma membrane. (Borg, Wolski, Gibbs, & O'Bryan, 2010)

1.2. Seminal Fluid

The seminal fluid, also known as semen, is composed by several components produced by different glands (Owen & Katz, 2005). The secretions produced by the accessory glands contributes to sperm survival and consequently to fertility. The secretions are mainly produced by the seminal vesicles (65%), prostate, bulbourethral glands and sometimes by epididymis and ampulla (30%). The sperm cells represent only 5% of the total semen volume; the volume of the human semen is around 3 mL and in healthy patients we can found 20 to 50 million sperm cells per milliliter (Rhoades & Bell, 2003; Owen & Katz, 2005). The secretions produced by the different organs are different in their composition. Secretions produced in the seminal vesicles are rich in fructose, which is the main energy source for ejaculated sperm, ascorbic acid and prostaglandins. These secretions produced by seminal vesicles are responsible for the coagulation of the semen after the ejaculation. The prostate fluid provides a milky aspect to the semen and is rich in acid phosphatase, citric acid, inositol, calcium, zinc and magnesium. The fibrinolysin is also produced in the prostate and it is responsible for liquefaction of the coagulated semen. Epididymis produces L-carnitine and neutral alpha-glucosidase, while ampulla produces a small amount of fructose (Owen & Katz, 2005; Rhoades & Bell, 2003; Hall, 2010). Finally, the bulbourethral glands are responsible for the production of the first portion

of semen that is released, called pre-ejaculate (Killick et al., 2011). This fluid is a viscous, clear, and salty liquid that neutralizes the acidity in the urethra. This fluid also provides some lubrication to the end of the penis, preparing it for sexual intercourse (Junqueira & Carneiro, 2004). The pre-ejaculate fluid is composed by large amounts of glutamyltransferase and acid phosphatase (Gohara, 1980).

The pH of the seminal fluid is around 7.5, because the alkaline secretions produced by the prostate neutralize the acidity of the secretions produced by the other organs (Hall, 2010).

1.3. Oxidative Stress and Reactive Oxygen Species

Infertility is a very common clinical situation, and it is classified by the World Health Organization (WHO) as a "disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (World Health Organization, 2013). About 15% of the couples suffer from this problem and half of these cases are directly or indirectly related with male infertility (Purvis & Christiansen, 1992; Agarwal & Prabakaran, 2005). The increase of oxidative stress (OS) is a possible etiology for this pathologic situation. Oxidative stress is a term used to define an imbalance between the levels of reactive oxygen species (ROS) and the levels of antioxidant systems, where the ROS are in excess (Agarwal & Prabakaran, 2005; Makker et al., 2009; Agarwal et al., 2008; Sharma et al., 2013; Chen et al., 2013).

The energy of the cell is mainly obtained by aerobic metabolism and by oxidative phosphorylation that occurs in mitochondria (Agarwal, Aitken, et al., 2012; Lavranos et al., 2012). However, during this process, the incomplete reduction of oxygen may occur leading to the formation of the superoxide anion ($O_2^{\bullet -}$) (Tremellen, 2008; Tvrdá et al., 2011). Reactive oxygen species can also be produced during the activity of some enzymatic systems, such as cytochrome P450 and xanthine oxidase (Aitken & Roman, 2008; Zangar et al., 2004). Reactive oxygen species include free radicals such as $O_2^{\bullet -}$ and hydroxyl radical ($^{\bullet}OH$), and non-radicals such as hydrogen peroxide, H_2O_2 (Kashou, Sharma, & Agarwal, 2013). However, ROS not only include oxygen derivatives, but also a subclass of nitrogen-containing compounds collectively known as reactive nitrogen species (RNS) that includes free radicals of nitrogen, such as nitric oxide (NO), peroxynitrite ($ONOO^-$), among others (Sikka, 2001; Makker et al., 2009; Tvrdá et al., 2011; Hamada et al., 2012; Doshi et al., 2012).

In spermatozoa, the most frequent ROS are $^{\bullet}OH$, peroxyl ($ROO^{\bullet}$) and $O_2^{\bullet-}$ radicals and H_2O_2, and they are relevant for cell's homeostasis (Makker et al., 2009; Agarwal, Aitken, et al., 2012).

1.3.1. Endogenous sources of ROS in seminal fluid

In fertile men, the ejaculate is usually constituted by several cellular types, such as mature spermatozoa, immature germ cells, leukocytes and Sertoli cells (Agarwal et al., 2008; Agarwal & Prabakaran, 2005; Chen et al., 2013). Among these cells, leukocytes (particularly neutrophils and macrophages) and abnormal or immature spermatozoa are the principal endogenous sources of ROS (Chen et al., 2013; Lavranos et al., 2012; Gavella et al., 1996) (Figure 2). The leukocytes are present throughout male genital tract, and can be found in normal human semen samples (Aitken, West, & Buckingham, 1994). These cells play an important role in infections, inflammations and in other cellular defense mechanisms, so in these conditions they are found in higher levels. Neutrophils, after being stimulated, produce large amounts of ROS, namely $O_2^{\bullet-}$ and H_2O_2 (Agarwal, Aitken, et al., 2012; Gharagozloo & Aitken, 2011). In samples with abnormal values of the basic parameters of spermatozoa analysis (such as concentration, motility and morphology), it is generally observed a higher rate of leukocytes and of abnormal spermatozoa, so an increase of OS is expected (Agarwal, Aitken, et al., 2012; Saleh et al., 2002).

According to WHO, by each semen milliliter we can found up to $1x10^6$ leukocytes with peroxidase-positive activity, and when this value is higher, it is considered leukocytospermia. Peroxidase-positive leukocytes are essentially represented by polymorphonuclear leukocytes that represent 50-60% of seminal leukocytes, and by macrophages that represent 20-30%. These leukocytes, when present in human semen, can be produced in prostate and/or in seminal vesicles (Keck et al., 1998; Makker et al., 2009; Agarwal et al., 2008; Saleh et al., 2002). Leukocytes, specifically neutrophils, have a specific enzyme in its granules called myeloperoxidase, and its activity contributes to a significant increase of ROS production in the organism. The oxidative damage of spermatozoa caused by ROS is generally associated with high concentrations of leukocytes in semen (Agarwal, Aitken, et al., 2012).

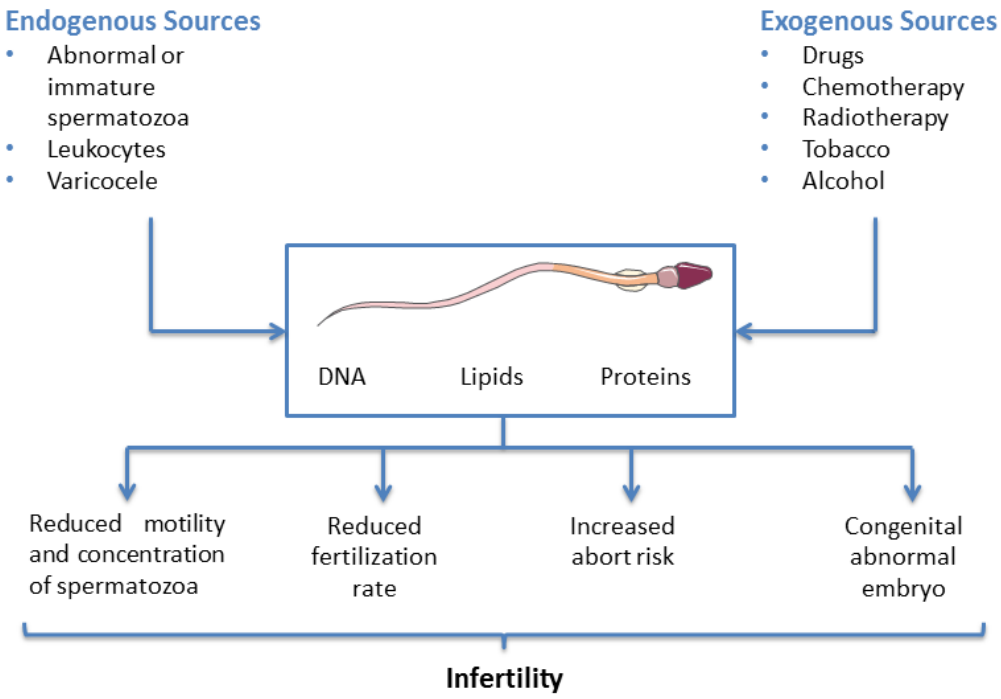

Figure 2 - Schematic representation of the sources of ROS, the molecules that are modified by the action of ROS and the consequences of those alterations.

During spermiogenesis, impairment of cytoplasmic extrusion mechanisms can occur with consequent release of spermatozoa with a higher quantity of residual cytoplasm in the germ epithelium. Apparently, these spermatozoa are immature and with functional anomalies, because they have a low binding capacity to the *zona pellucida* of the oocyte. The cytoplasm retention is directly related to the ROS formation by mechanisms mediated by the cytosolic enzyme glucose-6-phosphate dehydrogenase and by creatinine kinase (Makker et al., 2009; Agarwal et al., 2008). Immature and abnormal spermatozoa mitochondria also contribute to ROS formation, via complex I and III of the electron transport chain. The NOX5 enzyme (*nicotinamide adenine dinucleotide phosphate* (*NADPH*) *oxidase 5*) is expressed in the acrosome region and tail of human spermatozoon and it also appears to contribute to the increase of OS due to $O_2^{\bullet -}$ formation (Chen et al., 2013).

Varicocele is a common pathology in infertile men and it is also related to an increase in OS in spermatozoa. Varicocele is a tortuous distension of intrascrotal veins of the pampiniform plexus, and it is responsible for 15% of infertility cases (Naughton et al., 2001; Agarwal et al., 2008; Lavranos et al., 2012; Agarwal, Hamada, et al., 2012). Patients have increased levels of ROS in blood, testis and in ejaculated sperm, as well as increased levels of nitric oxide in spermatic veins (Agarwal, Aitken, et al., 2012; Agarwal & Said, 2005). The increase of ROS in

these patients is due to an excessive activity of xanthine oxidase, which leads to the production of $O_2^{\bullet-}$ and nitric oxide in dilated spermatic veins. These patients have also a reduced concentration of antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and vitamin C, in seminal plasma and in blood. Patients also have increased levels of 8-hidroxy-deoxyguanosine (8-OHdG), which indicates oxidative damage in DNA (Chen et al., 2004; Agarwal, Aitken, et al., 2012).

1.3.2. Exogenous sources of ROS in seminal fluid

Several environmental factors, as well as, lifestyle factors modulate the levels of ROS in seminal fluid. Certain drugs, such as aspirin or paracetamol, contribute to an increase in OS, due to stimulation cytochrome P450 activity, that is present in endoplasmic reticulum of hepatic cells, but it is also present in lungs, brain, kidneys and in the mitochondria of several cells, including spermatozoa (Agarwal & Said, 2005). Other drugs, such as SSRI (selective serotonin reuptake inhibitor) antidepressants, are responsible for a decrease in libido and consequently, for a decrease in the ejaculatory frequency (Corona et al., 2009). Thus, the spermatozoa are stored for a long time in epididymis, which leads to an increase of OS and then to a decrease in the integrity of biomolecules, such as DNA (Tanrikut et al., 2010). In cancer patients undergoing chemotherapy it is also observed an increase of OS. The administration of cyclophosphamide leads to an increase in the levels of testicular malondialdehyde (MDA, a marker of membrane damage) and to a decrease in the levels of CAT, an antioxidant enzyme of the testis; these alterations suggest an increase of OS. Radiotherapy used in oncologic treatment promotes a systemic inflammatory reaction that leads to an increase in OS mediated by leukocytes (Das et al., 2002; Ghosh et al., 2002; Agarwal, Aitken, et al., 2012).

Tobacco is one of the factors that has been closely related to the increase of seminal ROS levels and consequent DNA damage (Kumar, Kumari, & Murarka, 2009). Tobacco smoke has more than four thousand chemical compounds, and they are mainly ROS or RNS (Makker et al., 2009; Agarwal & Prabakaran, 2005). To prevent damages caused by molecular species, the organism expresses more antioxidant defense systems, such as MnSOD mitochondrial enzyme that is consequently increased in the human body. However, active smokers have a higher rate of DNA fragmentation and axoneme damages in spermatozoa and a decreased concentration of these cells (Agarwal, Aitken, et al., 2012).

Alcohol is also associated with an increase in OS in seminal fluid. The metabolism of ethanol induces an increase of respiratory chain activity, by increasing the production of NADH, with a consequent increase of ROS formation and with a commitment in ATP production (Agarwal & Prabakaran, 2005). During ethanol metabolism, acetaldehyde is formed inducing ROS formation through its interaction with proteins and lipids. Moreover, ethanol ingestion is related with an increase in the activity of xanthine oxidase and peroxisomal acyl-CoA oxidase, which contributes to OS (Nordmann, 1994; Agarwal, Aitken, et al., 2012). Alcohol can also be responsible for infertility by causing tissue damage (Agarwal & Prabakaran, 2005; Kumar et al., 2009). Testicular membranes are enriched in polyunsaturated fatty acids (PUFA) that are susceptible to peroxidation. Chronic ingestion of ethanol causes an increase of mitochondrial lipid peroxidation and a decrease in glutathione (GSH) levels in testis, that could be related to testis atrophy (Nordmann et al., 1990; Agarwal, Aitken, et al., 2012).

Previously, our laboratory has shown that acute lifestyle alteration (e.g. alcohol intake and cigarette smoking) result in a significant decrease in sperm concentration, total number of sperm cells in the ejaculate, volume of the ejaculate and a small decrease in motility (progressive and non-progressive). In turn, it was observed a significant increase in morphological abnormalities in the tail. Furthermore, the number of normal sperm cells and head abnormalities observed was not significantly different. In this study, it was proven that the alcohol consumption improves ROS production and reduces antioxidant concentration with a significant difference when it is associated with a poor nutrient diets (Ferreira et al., 2012).

1.4. ROS function in semen

1.4.1. Pathologic role

By promoting the oxidation of biomolecules (e.g. DNA, lipids and proteins) ROS may promote damage of sperm cells. The plasma membrane of spermatozoon is rich in PUFA, which makes it vulnerable to oxidation by ROS (Aitken, 1995; Kodama et al., 1996; Makker et al., 2009; Agarwal et al., 2008). The peroxidation of phospholipids of the plasma membrane promotes alterations in its fluidity, that causes a loss of motility and promotes a membrane fusion as well as a decrease of membrane enzymes and ionic channels activity, contributing to fertility impairment (Agarwal & Allamaneni, 2006; Tvrdá et al., 2011). Lipid peroxidation is a process

divided into three stages: initiation, propagation and termination. In the first stage, free radicals will interact with fatty acids chains, promoting the removal of hydrogen in neighbor methylene groups, forming water molecules and lipid free radicals, which can react with oxygen molecules to produce $ROO^{\bullet}$ radicals. During the propagation, these radicals can interact with other neighbor fatty acids to produce free radicals, thus propagating the reaction. Termination stage occurs when two radicals interact to produce a stable non radical product (Agarwal, Aitken, et al., 2012). A byproduct of lipid peroxidation is MDA, which is commonly analyzed in laboratories to measure peroxidative damage in spermatozoa (Aitken et al., 1989; Aitken & Fisher, 1994; Makker et al., 2009).

Reactive oxygen species are also responsible for causing damage in mitochondrial and nuclear DNA in human spermatozoa. These damages occur due to the action of these species on phosphodiester backbones and DNA bases, leading to its fragmentation (Wang, 2003; Gharagozloo & Aitken, 2011). Therefore, under normal conditions, antioxidant systems protect DNA of spermatozoon from small oxidative damages. However, if damages occur, the spermatozoa has no mechanisms to repair the DNA and so, it is very sensible to alterations promoted by ROS (Agarwal et al., 2007; Makker et al., 2009). Among these alterations, the most common are base modifications and deletions, , formation of frameshifts and crosslinks of DNA and chromosomal rearrangements (Kemal Duru et al., 2000; Agarwal et al., 2008; Tvrdá et al., 2011). In turn, at fertilization, the oocyte has mechanisms to repair sperm DNA (Agarwal et al., 2007; Makker et al., 2009).

Reactive oxygen species can also cause damages in proteins, interfering with enzymatic activity or with structural proteins function. For example, H_2O_2 seems to inhibit enzymes activity, such as glucose-6-phosphate-desidrogenase (G6PD). This enzyme controls the flow of glucose due to hexose monophosphate shunt, which in turn, controls the intracellular concentration of NADPH (Aitken et al., 1997; Makker et al., 2009; Agarwal et al., 2008). Highly reactive and stable products, such as protein hydroperoxide, can also be formed through the oxidation of these biomolecules by ROS and RNS. Although the oxidized proteins are functionally inactive and quickly removed, some may accumulate in the organism causing damages related to aging and pathologies (Agarwal, Aitken, et al., 2012).

The excessive production of nitric oxide (NO) can cause protein nitration in tyrosine residues. Tyrosine nitration occurs when a NO_2 group is added at the third position of either free or protein-bound tyrosine, leading to the formation of 3-nitrotyrosine (3-NT) (Sultana & Butterfield, 2008). Nitric oxide is produced by the catalytic conversion of arginine to citrulline

by nitric oxide synthases (NOS) (Sultana & Butterfield, 2008; Michel & Feron, 1997). Nitric oxide and $O_2^{\bullet-}$ react with each other, in the presence of CO_2, producing peroxynitrite ($ONOO^-$), which is a product highly reactive with biomolecules such as proteins, lipids, and carbohydrates. The reaction of peroxynitrite with proteins can result in S-nitrosylation or in the production of 3-NT (Butterfield et al., 2007; Butterfield & Kanski, 2001; Sultana & Butterfield, 2008). 3-nitrotyrosine corresponds to a covalent protein modification and has been used as a marker of nitrosative stress. The nitration of proteins, such as mitochondrial and Cu/Zn SOD, actin and tyrosine hydroxylase, may result in their inactivation (Butterfield et al., 2007).

Reactive oxygen species can also promote a decrease in the number of spermatozoa by activating apoptosis. Apoptosis is a fundamental process for the elimination of abnormal germ cells that are produced during spermatogenesis. Mitochondrial membrane fluidity alterations promoted by ROS can induce apoptosis by releasing cytochrome c into the cytosol with consequent activation of caspases. Effectively, recent studies demonstrated that in patients with male factor infertility, with increased sperm damage by ROS, caspases-3 and -9 are activated in human ejaculated sperm (Makker et al., 2009; Agarwal et al., 2008; Tvrdá et al., 2011; Agarwal & Said, 2005; Wang, 2003). Mitochondrial membrane fluidity alterations also allow the release of apoptosis inducing factor (AIF) from this organelle. This mediator promotes apoptosis by a mechanism independent from caspases, by interacting with DNA and promoting its fragmentation (Paasch et al., 2004; Candé et al., 2002; Makker et al., 2009).

1.4.2. Physiologic role

Reactive oxygen species do not play just a pathologic role in our organism (Figure 3). Currently, it is believed that these molecular species have a physiological role when they are in an ideal concentration (Aitken, 1997). Low ROS level regulates fertilization, acrosome reaction, hyperactivation, motility and capacitation. Actually, recent studies demonstrated that incubation of spermatozoa with low concentrations of H_2O_2 stimulate spermatozoon capacitation, hyperactivation, acrosome reaction and oocyte fusion. On the other hand, nitric oxide and $O_2^{\bullet-}$ also seem capable of promoting capacitation and acrosome reaction (Makker et al., 2009; Agarwal et al., 2008; Agarwal, Aitken, et al., 2012; Mahfouz et al., 2010).

The aim of capacitation is to prepare spermatozoon to its interaction with oocyte, which occurs in the female genital tract. In this process, there is an increase of the intracellular levels of calcium, ROS and tyrosine kinase, which, in turn, are responsible for the increase of cyclic

adenosine monophosphate (cAMP). The increase in these molecules will promote the tyrosine phosphorylation by the activation of different signaling pathways. In turn, the tyrosine phosphorylation is responsible for the hyperactivation of spermatozoa, a process that leads to an alteration of motility, where the weak and wavy movement is replaced by a stronger movement. This process allows spermatozoa to initiate the acrosome reaction, facilitating the interaction between spermatozoon and oocyte (de Lamirande et al., 1997; Visconti et al., 1995; Makker et al., 2009; Agarwal, Aitken, et al., 2012).

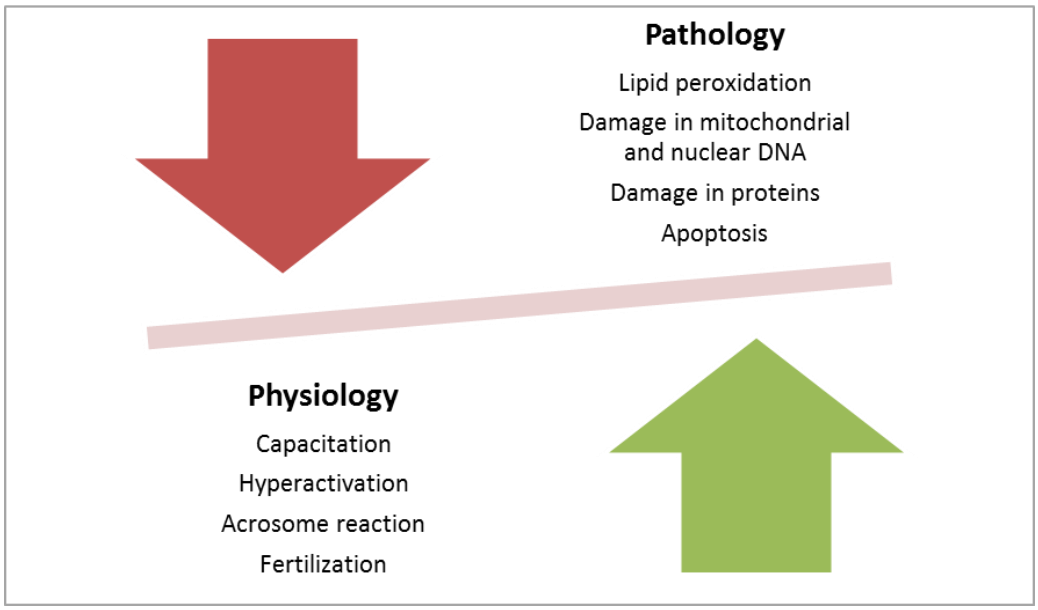

Figure 3 - The pathologic and physiologic roles of ROS.

1.5. Antioxidant defenses

The human organism has antioxidant defense systems that control the ROS action (Table 1). These defense mechanisms are also localized in seminal plasma and spermatozoa. Seminal plasma has enzymatic antioxidant systems, such as SOD, CAT, GPx and non-enzymatic antioxidants, such as vitamins A, C and E, glutathione, among others (Tvrdá et al., 2011).

Table 1- Overview of antioxidant species in male reproductive tract. The table summarizes the antioxidant species that are present in some regions of the male reproductive system and describes its functions.

Antioxidant species	Function	Localization	References
Superoxide Dismutase (SOD) • **MnSOD** • **CuZnSOD** • **ExSOD**	Conversion of $O_2^{\bullet-}$ in H_2O_2 and O_2	Mitochondrial matrix (MnSOD), cytoplasm (CuZnSOD), Sertoli cells and germ cells (extracellular SOD)	(Fridovich 1995; Okado-Matsumoto & Fridovich 2001)
Glutathione Peroxidase (GPx) • **GPx1** • **GPx4** • **GPx5**	Reduction of H_2O_2 in H_2O	Mitochondria and nucleus (GPx1 and GPx4), acrosome region (GPx1), Leydig cells (GPx4) and epididymis (GPx4 and GPx5)	(Agarwal, Aitken, et al., 2012)
Catalase (CAT)	Reduction of H_2O_2 in O_2 and H_2O	Cytoplasm, acrosome of spermatozoa and seminal plasma (from prostate)	(Jeulin, Soufir, Weber, Laval-Martin, & Calvayrac, 1989)
Vitamin E (α-tocoferol)	Elimination of $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$	Sertoli cells, spermatozoa membrane, spermatids	(Suleiman, Ali, Zaki, El-Malik, & Nasr, 1996)
Vitamin C	Neutralization of $^{\bullet}OH$, $O_2^{\bullet-}$ and H_2O_2 action; prevention of lipid peroxidation, contribution to the vitamin E regeneration and protection of DNA from H_2O_2 damage	Seminal plasma	(Makker et al., 2009)
Cytochrome C	Removal of $O_2^{\bullet-}$, stimulation of apoptosis that allows the elimination of damaged germ cells	Mitochondrial intermembrane space	(Agarwal, Aitken, et al., 2012)
Coenzyme Q10	Prevention of oxidative and nitrative stress, by removing free radicals and inhibiting lipid peroxidation and inflammation	Middle piece of spermatozoa	(Karbownik et al. 2001; Alvarez & Storey 1995)
Melatonin	Prevent oxidative stress	Testis	(R John Aitken & Roman, 2008)
Taurine	Overexpression of antioxidant enzymes and prevents ROS formation in mitochondria	Cauda epididymis	(Aruoma et al. 1988; Schaffer et al. 2009)
Glutathione (GSH)	Reacts with cytotoxic aldehydes	Extracellular space	(Sørensen, Stoltenberg, Danscher, & Ernst, 1999)

N-acetylcysteine (NAC)	Glutathione (GSH) synthesis	Seminiferous tubules	(Agarwal, Aitken, et al., 2012)
Albumin	Prevents the production of free radicals and traps free radicals	Seminal plasma	(Roche, Rondeau, Singh, Tarnus, & Bourdon, 2008)

Superoxide dismutase catalyzes $O_2^{\bullet-}$ conversion in H_2O_2 and O_2 (Fridovich, 1995). Superoxide dismutase has two different isoforms present in cells. An isoform of SOD, conjugated with manganese in its active site (MnSOD), is localized in the mitochondrial matrix. This isoform acts in $O_2^{\bullet-}$ produced in the mitochondria (Fridovich, 1995). Another isoform of SOD, conjugated with copper and zinc (CuZnSOD), is localized in the cytoplasm (Okado-Matsumoto & Fridovich, 2001). There is another isoform of SOD, extracellular SOD, that is produced by Sertoli cells and germ cells (Agarwal, Aitken, et al., 2012; Tvrdá et al., 2011). Germ cells can induce the secretion of this isoform in a process mediated by the action of several cytokines, such as interleukine-1a (Aitken & Roman, 2008).

Glutathione peroxidase is a selenoenzyme type that catalyzes the reduction of H_2O_2 in H_2O, with the concomitant oxidation of glutathione (Agarwal, Aitken, et al., 2012). In mature spermatozoa, GPx is localized in mitochondria, nucleus and acrosome region (Tvrdá et al., 2011). In testis, there are several isoforms being the most common the phospholipid hydroperoxide (PHGPx or GPx4), that is highly expressed in sperm cells and in Leydig cells (R John Aitken & Roman, 2008). Another isoform, GPx5, was found in the mouse epididymis. This isoform is secreted in the proximal segment of the caput, being also found in the lumen of the corpus and cauda epididymis (Vernet et al., 1997).

Catalase is an enzyme that catalyzes reduction of H_2O_2 into O_2 and H_2O, completing the action of SOD (Tvrdá et al., 2011; Garrido et al., 2004). However, the presence of this enzyme is still controversial, because it is usually associated with the possibility of contamination (Jeulin et al., 1989; Tramer et al., 1998). Nevertheless, some studies showed its presence in human and rat spermatozoa, in the cytoplasm and acrosome in low concentration and also in the seminal plasma. This enzyme is absent in spermatozoa of other mammalian species (Agarwal, Aitken, et al., 2012; Jeulin et al., 1989; Tramer et al., 1998). The seminal catalase is produced in the prostate (Jeulin et al., 1989).

Vitamin E (α-tocoferol) is present in large amounts in Sertoli cells and in the spermatozoa membrane, and it may also be found in lower concentrations in spermatids (Suleiman et al.,

1996; Aitken & Roman, 2008). Its function is to eliminate $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ and it is directly correlated with the dose (Suleiman et al., 1996; Makker et al., 2009; Agarwal et al., 2008; Tvrdá et al., 2011; Lombardo et al., 2011). Vitamin C neutralizes $^{\bullet}OH$, $O_2^{\bullet-}$ and H_2O_2 action, preventing the agglutination of spermatozoa (Makker et al., 2009; Agarwal et al., 2008). Furthermore, vitamin C also helps to prevent lipid peroxidation, contributes to the vitamin E regeneration and protects DNA from H_2O_2 damage (Tvrdá et al., 2011). Vitamin C, due to reductase dehydroascorbate dependent-GSH action that is present in high concentration in testis, remains in its reduced state. When the concentration of vitamin C and E in the organism is low, spermatogenesis and testosterone production can be affected due to an increase of OS (Aitken & Roman, 2008).

Cytochrome C, which is localized in the mitochondrial intermembrane space, has a double function in the removal of $O_2^{\bullet-}$. The cytochrome is reduced by an electron of the electron transporter chain or by $O_2^{\bullet-}$. After undergoing reduction, cytochrome C is oxidized by cytochrome C oxidase (Agarwal, Aitken, et al., 2012). Additionally, cytochrome C stimulates apoptosis allowing the elimination of damaged germ cells, contributing to an extra protection of the testis (Aitken & Roman, 2008). In turn, coenzyme Q10 is a non-enzymatic antioxidant localized in the middle piece of spermatozoon, protecting it from oxidative damage (Alvarez & Storey, 1995; Karbownik et al., 2001; Makker et al., 2009; Agarwal et al., 2008;Tvrdá et al., 2011). Coenzyme Q10 is a component of mitochondrial respiratory chain, playing a role in energetic metabolism and as liposoluble antioxidant. This molecule reverts endothelial dysfunction preventing oxidative and nitrative stress, removing free radicals and inhibiting lipid peroxidation and inflammation. Its biosynthesis is very active in testis (Agarwal, Aitken, et al., 2012).

Another antioxidant present in the organism is melatonin (N-acetyl-5-methoxytryptamine), produced by the pineal gland. This molecule helps to prevent OS in testis. Melatonin is soluble in the aqueous medium and in the lipid medium, so it can easily cross the blood testis barrier, thus protecting germ epithelium. The decrease of melatonin levels is common in infertile men and is associated with the reduction of motility, leukocytospermia, varicocele and non-obstructive azoospermia (Aitken & Roman, 2008).

Taurine, a free amino acid present in several tissues, can also play an antioxidant function (Wright et al., 1986). This amino acid is usually found in the cauda epididymis. Although it cannot directly eliminate ROS, it inhibits their production (Aruoma et al., 1988; Schaffer et al., 2009). Taurine seems to act by different forms, namely inducing overexpression of antioxidant

enzymes, mainly SOD and GPx and preventing ROS formation in mitochondria due to calcium accumulation. Actually, taurine interferes with excessive charge of calcium, due to the stimulation of Na^+/Ca^{2+} exchanger (Schaffer et al., 2009; Wu et al., 2005). To attenuate mitochondrial ROS formation, taurine is conjugated with mitochondrial tRNA, facilitating translation of mitochondrial proteins and a correct energy production. In the absence of this amino acid, dysregulation of electron carrier chain occurs, with consequent effect in ATP production and in $O_2^{\bullet-}$ formation (Agarwal, Aitken, et al., 2012; Schaffer et al., 2009; Suzuki et al., 2002).

Glutathione (GSH) is a tripeptide that is the most important and abundant nonenzymatic scavenger in and outside cells. It has a cysteine residue that provides a reactive thiol group (SH) and this group can interact with free radicals (Agarwal, Aitken, et al., 2012). Glutathione is present in extracellular space, where it is able to react with cytotoxic aldehydes, which were produced during lipid peroxidation. This reaction protects the sperm plasma membrane against ROS (Sørensen et al., 1999).

N-acetylcysteine (NAC) is a metabolite of L-cysteine involved in GSH synthesis that is important in ROS neutralization. This antioxidant plays an important role in the seminiferous tubules, where it contributes to the survival of germ cells (Agarwal, Aitken, et al., 2012).

Albumin is the major component of the epididymis fluid, and it has two mechanisms to decrease the OS (Roche et al., 2008). Albumin binds metal ions (e.g. copper and iron), so it prevents the production of free radicals (Halliwell, 1988). In other way, albumin is involved in free radical trapping being this action mediated by a sulfhydryl group (Cys34) that has the ability to form disulfide with several compounds. Thus, through this group, albumin may scavenge hydroxyl radicals as well as RNS (Roche et al., 2008).

In general, the reduction of spermatozoa motility promoted by the increase of OS can be prevented by antioxidant systems such as vitamin C and E, GSH, SOD, CAT, albumin and taurine. N-acetylcysteine and coenzyme Q10 promote an increase in spermatozoa motility (Makker et al., 2009; Agarwal et al., 2008).

1.6. Evaluation of oxidative stress in seminal fluid

To evaluate OS in a human semen sample, several methods can be used (Figure 4) and are based in:

a) direct measure of ROS levels;
b) measure of biomolecules damage and
c) detection of antioxidants levels.

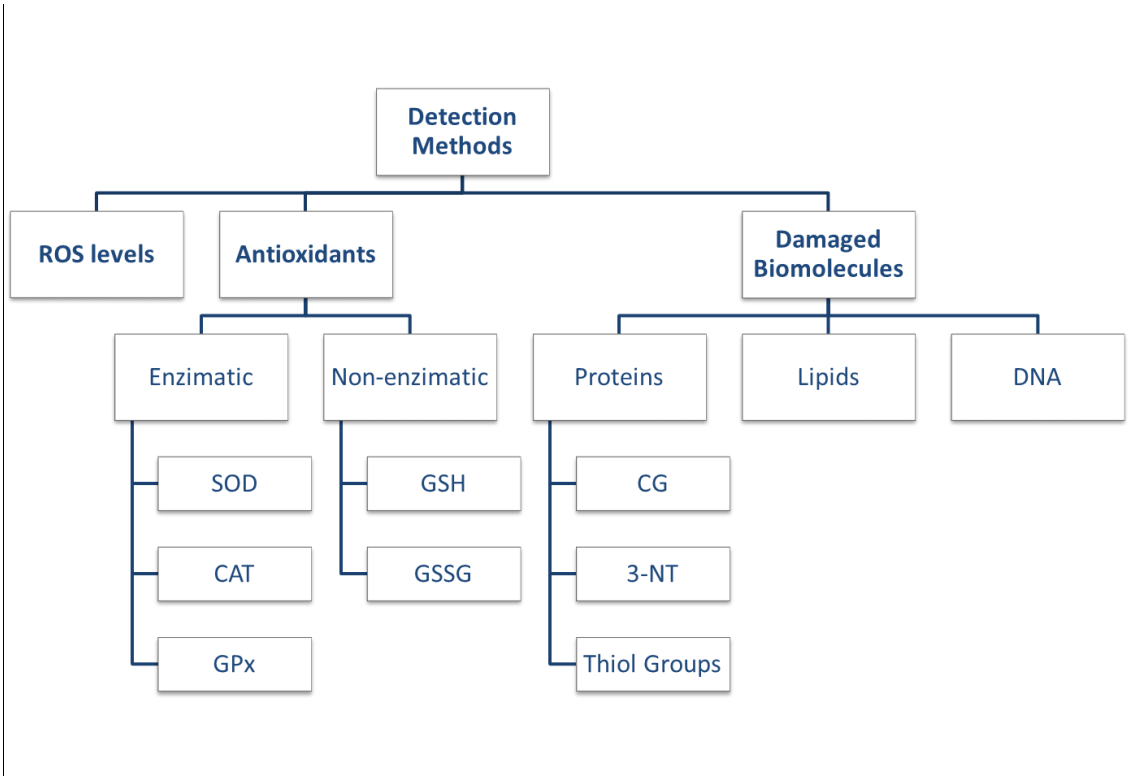

Figure 4 - Schematic representation of the detection methods to measure oxidative stress.

3-NT: 3-Nitrotyrosine; CAT: Catalase; CG: Carbonyl Groups; DNA: Deoxyribonucleic Acid; GPx: Glutathione Peroxidase; GSH: Reduced Glutathione; GSSG: Oxidized Glutathione; ROS: Reactive Oxygen Species; SOD: Superoxide Dismutase.

Of these, direct measurement of ROS is the preferred method but given the methodological difficulties inherent to the instability of ROS, it is rarely used experimentally. Thus, the evaluation of the damage triggered by ROS in proteins, lipids or DNA, as well as, the evaluation of activity of antioxidant defense systems constitutes the most frequent methods to indirectly detect OS (Tvrdá et al., 2011). Between antioxidant defense systems, SOD and CAT activities are the most frequently used (Tvrdá et al., 2011).

The direct measurement of ROS levels can be performed by different methods. ROS levels present in cellular membrane surface can be measured by reactions with nitroblue tetrazolium

or with cytochrome-C-Fe^{3+} (Tvrdá et al., 2011; Aitken et al., 1992). Electron spin resonance is a very sensitive method and it allows identifying ROS type produced in the cell. However, the involved equipment and the results interpretation difficult the performance of this technique to ROS quantification in seminal fluid (Tvrdá et al., 2011; Sikka, 1996).

Another method consists in the analysis of reactions between ROS and luminol by luminescence (Sikka, 1996). Chemiluminescence detection methods, such as luminol and lucigenin, can be used to quantify the redox activity of spermatozoa. Luminol is a very used substrate because it is highly sensitive, and it is capable to react with several ROS at neutral pH. Between the sensible ROS to this method are H_2O_2 and $O_2^{\bullet-}$, because CAT and SOD can promote a reduction in the chemiluminescence signal, and these antioxidant systems reduce the concentration of these species (Agarwal, Aitken, et al., 2012; Kashou et al., 2013; Aitken et al., 1992). H_2O_2 and endogenous peroxidase are responsible for the induction of chemiluminescence in spermatozoa, through the oxidation of an electron. The reaction of luminol with ROS is responsible for a signal production that can be quantified in a luminometer. The result is usually showed in $x10^6$ photons per minute by $20x10^6$ spermatozoa (Agarwal, Aitken, et al., 2012). In turn, the technique that uses lucigenin is more specific to $O_2^{\bullet-}$, verifying a chemiluminescence signal reduction, due to SOD action on the O_2^- (Aitken et al., 1992). The main difference between this method and luminol method is that lucigenin need to be reduced by an electron to be activated. This reduction will form a radical that readily gives its electron to a molecule of O_2 to form $O_2^{\bullet-}$. The posterior interaction between lucigenin and $O_2^{\bullet-}$ leads to the formation of dioxetane that emits luminescence in its decomposition (Agarwal, Aitken, et al., 2012; Liochev & Fridovich, 1997). Luminol is used more frequently than the lucigenin, because it allows evaluating intra- and extracellular ROS, and lucigenin only allows detecting $O_2^{\bullet-}$ that is present in extracellular medium. However, both detection methods can be performed simultaneously, thus allowing to evaluate with more precision intra and extracellular ROS concentration (Tvrdá et al., 2011).

Apart from these tests, the products of the action of ROS on biomolecules can be further evaluated. Between the most common protein oxidation markers, we can found carbonyl and thiol groups and 3-NT. Reactive oxygen species can act on proteins, because its side chains are very susceptible to the action of these species, leading to carbonyl groups production (Rodrigo & Toro, 2009). The evaluation of oxidized proteins through this marker in spermatozoa and in seminal fluid is evaluated spectrophotometrically or by immunoblotting by measuring the formed product with 2,4-dinitrophenylhydrazine (DNPH) (Lenz et al., 1989; Robinson et al.,

1999). This evaluation by immunoblotting is also performed to measure the presence of 3-NT (Sultana & Butterfield, 2008).

The spermatozoa protein oxidation by ROS induces loss of thiol groups, leading to a reduction of SOD activity, with a consequent increase of the concentration of $O_2^{\bullet-}$, promoting a decrease in motility. The evaluation of thiol groups can be made through reactions between these groups and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), which forms a colored product that can be measured spectrophotometrically (Shiva et al., 2011).

Reactive oxygen species levels can be indirectly analyzed by malondialdehyde (MDA) levels, a marker of lipid peroxidation (Esteves & Agarwal, 2011). Malondialdehyde levels inversely correlate with spermatozoon motility and with fusion potential of spermatozoa and oocytes (Aitken, Harkiss, & Buckingham, 1993). This assay is utile in the determination of ROS levels before inherent procedures of assisted reproduction and in diagnosis of infertile patients (Oral et al., 2006; Agarwal, Aitken, et al., 2012). Malondialdehyde formation can be evaluated spectrophotometrically by reaction with thiobarbituric acid (Zarghami & Khosrowbeygi, 2005; Agarwal, Aitken, et al., 2012).

8-hydroxy-2'-deoxyguanosine molecule (8-OHdG) is a marker of DNA damage induced by OS (Garrido et al., 2004). Elevated levels of 8-OHdG are associated with reduced fertilization rates (Agarwal & Saleh, 2002). Additionally, the 8-OHdG presence is inversely proportional to the antioxidant levels in blood plasma. The test to evaluate 8-OHdG is relatively simple, and it can be performed using the ELISA method (Agarwal, Aitken, et al., 2012).

Glutathione is a tripeptide and it is the most abundant non-protein thiol in sperm cells and is important for maintaining the intracellular redox state (Agarwal, Aitken, et al., 2012; Irvine 1996). Its concentration can also be measured spectrophotometrically by reaction with DTNB (Abeydeera et al., 1998).

Catalase reduces H_2O_2 into H_2O and O_2 and this elimination of H_2O_2 can be measured directly by the decrease in the absorbance at 240 nm. The catalase activity is calculated from the change in absorbance and expressed as U/ml (Li & Schellhorn, 2007). SOD is responsible for the conversion of $O_2^{\bullet-}$ in H_2O_2 and O_2, and its activity can be measured by colorimetric assay, being expressed as U/mL (Zelen et al., 2010).

Glutathione Peroxidase is an antioxidant protein that reduces H_2O_2 into H_2O and O_2. The activity of this protein can be assayed spectrophotometrically and this method is based on the ability of GPx to catalyze the oxidation of glutathione by hydroperoxide (Vaisberg et al., 2005).

1.7. Oxidative stress evidences in semen analysis

The routine laboratorial tests performed in human semen (spermogram) do not directly evaluate OS; however, some altered parameters in the ejaculate can reflect the action of ROS (Table 2). Thus, the increase of OS can contribute to hiperviscosity of semen due to an increase of MDA levels and to a decrease of antioxidant plasmatic levels. Furthermore, the semen shows a slight discoloration and its pH increases to values higher than 8. The result of the spermogram shows a higher number of rounded cells that can represent leukocytospermia, or an increased number of immature spermatozoa. Spermatozoa can have a reduced motility and they can also show an increase in the number of morphologically altered spermatozoa, with an increase of residual cytoplasm (Agarwal, Aitken, et al., 2012). The hypo-osmotic swelling test (HOST) allows to visualize a decrease of spermatozoa membrane integrity that can be associated to an increase of lipid peroxidation promoted by ROS (Dandekar et al., 2002; Agarwal, Aitken, et al., 2012).

Table 2- Spermogram parameters that are altered due to ROS action.

Parameter	Alteration
Viscosity	Increased
Coloration	Discolored
pH	Higher than 8
Round cells	Leukocytospermia and increased number of immature spermatozoa
Motility	Reduced
Morphology	Altered and increase of residual cytoplasm
Membrane	Poor membrane integrity

2. AIMS

The aims of this thesis were

1. to analyze the influence of the lifestyle alterations made during academic festivities in male reproductive health, namely in the antioxidant capacity, and
2. to analyze the correlation between the oxidative balance and the clinical parameters of sperm cells.

Previous studies analyzed the total antioxidant capacity of the seminal fluid, however this study focused in the total antioxidant capacity of the sperm cells. The OS damage in the biomolecules of the sperm cells, namely the proteins, was also evaluated.

This study included the:

- Human sperm samples collection and preparation;
- Evaluation of seminal quality, according to WHO guidelines;
- Measurement of the total antioxidant capacity of the sperm cells;
- Detection of specific antioxidants usually found in the sperm cells;
- Evaluation of ROS effect on spermatozoa proteins.

3. METHODS

3.1. Semen Samples Origin

The samples used in this thesis were obtained from two previous projects: the project for the "Associação Portuguesa de Urologia" and the project "*Para o Frasco*" from 2010 and 2011.

3.1.1. Project "*Associação Portuguesa de Urologia – APU*"

Samples of semen from the project "*Criação de plataforma para avaliar a fertilidade masculina" of "Associação Portuguesa de Urologia (APU)*" were used to determine the total concentration of antioxidants in the sperm cells and to evaluate the presence of GPx4 and SOD antioxidant proteins in these cells. In this thesis, we also evaluated the total antioxidant capacity that is present in the seminal plasma. Then, we also proceeded to the evaluation of the action of ROS on proteins of the spermatozoa, by determining the presence of 3-NT and the production of carbonyl groups. This project from *APU* aims to create a platform of diagnostic that will allow to easily determining apoptotic levels of a biological sample. The preparation of these samples and the tests performed with them are present in the Figure 5.

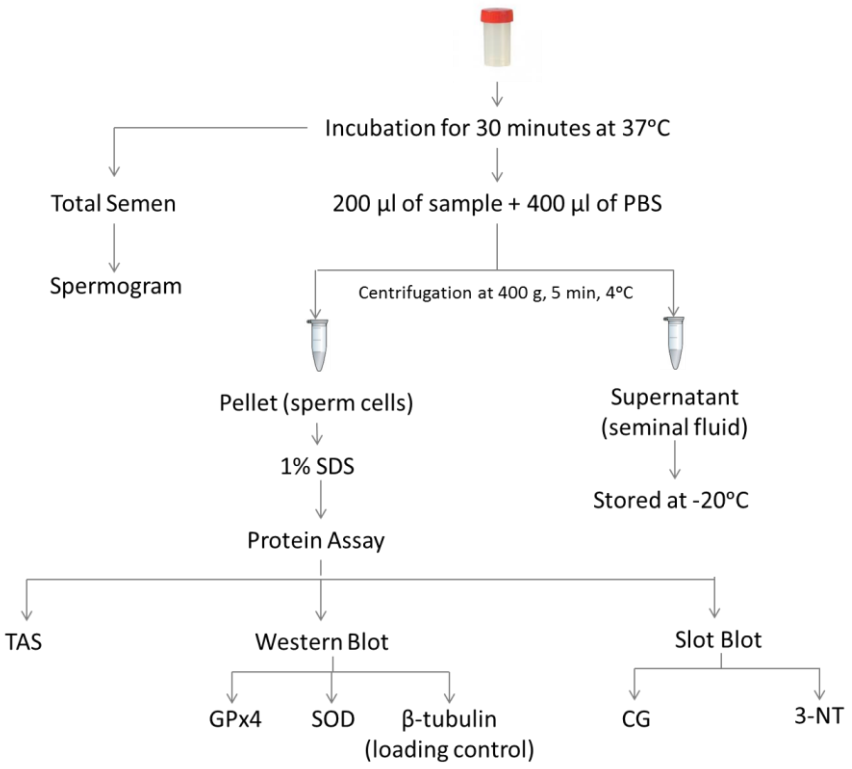

Figure 5- Sample workflow from the *APU* project that was used in this thesis.

3-NT: 3-Nitrotyrosine; CG: Carbonyl Groups; GPx4: Glutathione Peroxidase 4; PBS: Phosphate Buffered Saline; SDS: Sodium Dodecyl Sulfate; SOD: Superoxide Dismutase; TAS: Total Antioxidant Status.

The samples were first centrifuged, and the pellets obtained (corresponding to the spermatozoa fraction) were lysed in 1% SDS and a protein assay performed (described in the section 3.5). Next, we performed the total antioxidant status (TAS) assay (described in the section 3.6) to evaluate the antioxidant capacity of these sperm samples and Western Blotting to evaluate the presence of the antioxidant proteins, SOD and GPx4 (described in the section 3.7). Lastly, we performed two slot blots to determine the presence of 3-NT and carbonyl groups (described in the section 3.8).

3.1.2. Project "*Para o Frasco 2010/2011*"

Semen samples aliquots from "*Para o Frasco"* 2010 and 2011 were also used in this thesis to study the total antioxidant capacity and to evaluate the presence of specific antioxidants (GPx4 and CuZnSOD) and 3-NT in the spermatozoa.

"*Para o Frasco*" was a study that evaluated the influence of acute lifestyle changes during the academic festivities, such as the consumption of alcohol, tobacco and drugs, in the male fertility. The samples were given by young male volunteers, in reproductive age (more than 18 years), at University of Aveiro, during three defined different moments: the first moment (time point 1 – TP1) was before the academic week, the second moment (time point 2 – TP2) was one week after the academic festivities and the third moment (time point 3 – TP3) was around three months after this festivity.

When the volunteers delivered their samples, in TP1, they answered to a questionnaire (questionnaire 1- see section 8.2.1) where the habitual drug, alcohol and tobacco consumption as well as their sexual abstinence and the history of diseases were asked. At the second sample delivery, the volunteers answered to a new questionnaire (questionnaire 2 – see section 8.2.2) where the questions were identical, but the consumption questions were about the academic festivities. They also answered to a questionnaire at TP3 about their consumption during the last three months (questionnaire 3 – see section 8.2.3).

When the thesis began, the samples were already processed as a pellet, which was then lysed in 1% SDS. Then, as we did with "*APU*" samples, it was performed a protein assay, evaluated the TAS as well as the presence of SOD, GPx4 and 3-NT.

3.2. Semen sample delivery

The samples of semen were collected to a sterile recipient by masturbation according to the WHO recommendations, with a sexual abstinence during a minimum of 2 days and a maximum of 7 days. After the collection, the samples were maintained at room temperature (RT) during a maximum of one hour, until be delivered to the laboratory. Then, the samples were allowed to liquefy by incubation at 37°C for approximately 30 minutes.

3.3. Preparation of Samples

After the liquefaction, the spermogram was performed in one aliquot of 200 µL of the sample. The spermogram was performed based on macroscopic and microscopic examinations, according to WHO guidelines.

Then, semen samples were separated in aliquots of 200 µL of semen with 400 µL of phosphate buffered saline (PBS). Then, the seminal plasma and spermatozoa were separated by a centrifugation during 5 minutes, 400 g at 4°C. After this step, the supernatant (seminal plasma) was frozen at -20°C and stored. The pellet obtained during the centrifugation, which corresponds to the spermatozoa, was washed and then centrifuged two more times with PBS. The pellet was then separated and frozen at -20°C with 1% SDS.

3.4. Seminal Analysis

3.4.1. Macroscopic Examination

The macroscopic examination began soon after liquefaction, to prevent dehydration or changes in temperature that will affect the semen quality.

3.4.1.1. Liquefaction

After the ejaculation, the semen is typically a semisolid coagulated mass. After a few minutes at RT, the semen initiates the liquefaction that leads to a more homogeneous and quite watery sample. In the final stages only small areas of coagulation can be seen. The process of liquefaction usually takes 15 minutes at RT; however, in some cases it can take up to 60

minutes or even more. When the liquefaction takes more than 60 minutes, this situation should be registered.

3.4.1.2. Viscosity

After liquefaction, it was analyzed the viscosity of the sample. This parameter can be analyzed by aspirating the sample using a micropipette and allowing the semen to drop by gravity observing the length of any thread. When the viscosity is normal, the sample is dispensed by micropipettes in small discrete drops. An abnormal sample, the sample form drops with a thread with more than 2 cm long.

3.4.1.3. Appearance and Volume

A normal sample, that is liquefied, shows a homogeneous and grey-opalescent appearance. When the semen sample has a low sperm concentration, it appears less opaque. The color of the sample may also have some alterations: when the red blood cells are present, the sample has a red-brown color; the samples of men with jaundice or taking certain vitamins or drugs may show a yellow color.

To measure the volume, we transferred the sample from the container to a falcon of 15 mL and read the volume directly from the graduations.

3.4.2. Microscopic Examination

The microscopic examination includes the analysis of motility, concentration and morphology of the spermatozoa. These parameters were performed based on WHO guidelines.

3.4.2.1. Aggregation and Agglutination

Aggregation refers to the adherence of non-motile spermatozoa to each other or even to the adherence between motile spermatozoa and mucus strands, non-sperm cells as well as debris.

In turn, agglutination refers to the adherence between several motile spermatozoa; this interaction might be head to head, tail to tail or mixed. In the majority of the cases, the motility is present with a shaking motion; however, when the sample is very agglutinated the motion is limited. There are four grades of agglutination:

Grade 1 – less than 10 spermatozoa per agglutinate with many free spermatozoa

Grade 2 – 10 to 50 spermatozoa per agglutinate with free spermatozoa

Grade 3 – more than 50 spermatozoa per agglutinate with few free spermatozoa

Grade 4 – all spermatozoa are connected.

3.4.2.2. Concentration

The sperm concentrations that can be measured are between $2x10^6$ and $50x10^6$ and the samples with a higher concentration needed to be diluted. The evaluation was performed in this range to decrease the presence of lecture errors. First, we mixed the liquefied semen sample and put on a glass slide covered with a coverslip to determinate the appropriate dilution. Then, the appropriate volume of sample was added to a fixative (composed of 5 g of $NaHCO_3$ and 1 mL of 35% (v/v) formalin in 100 mL of distilled water). This mixture was then vortexed for around 10 seconds and the improved Neubauer chamber filled with 10 µL of the mixture. The chamber was analyzed at 400x magnification, counting at least 200 spermatozoa. Initially, the central grid (number 5) of one side was assessed. Then, the concentration of spermatozoa per milliliter was determinate using the following formula: C = (N/n) x (1/20) x dilution factor; N is the number of spermatozoa, n is the volume of the total number of rows examined (20 ηL each for grid number 5). Lastly, the total number of spermatozoa in ejaculate was determined by the multiplication of sperm concentration by the semen volume.

Table 3 – Semen dilutions used for the determination of sperm concentration.

Spermatozoa per x400 field	Dilution required	Semen (µL)	Fixative (µL)
>101	1:20	50	950
16-100	1:5	50	200
<15	1:2	50	50

3.4.2.3. Motility

The sperm motility was analyzed as soon as possible after liquefaction, preferably 30 minutes, to limit the deleterious effects of dehydration, pH or changes in temperature on motility. The sperm motility could be assessed on samples with sperm concentrations between $2x10^6$ and $50x10^6$ spermatozoa. To evaluate the sperm motility we first mixed well the semen sample, removing immediately an aliquot. Then, we prepared a wet preparation; this preparation must had approximately 20µm deep , and requires a volume of 10µL. After the application of the sample, we waited around 60 seconds for the sample to stop drifting. We proceeded to the

slide examination with a phase-contrast optics at x400 magnification. The percentage of the different motile categories (motile progressive, motile non-progressive and immotile) were assessed in approximately 200 spermatozoa.

3.4.2.4. Morphology

To determine the sperm morphology, we prepared a smear of semen and the spermatozoa were then assessed for the percentage of normal vs abnormal forms. The abnormalities of the spermatozoa can refer to head, midpiece and tail defects as well as an excess of residual cytoplasm. A semen smear was prepared by application of 10 µL of semen to the end of the slide. A coverslide was used to pull the drop of semen along the surface of the slide. The slides were then allowed to dry in air before fixation. This next step was performed with methanol 95% during 1 hour. The staining was performed in two steps: the cytoplasm staining was performed with eosin during 10 seconds, conferring a pink coloration; in turn, the nucleus was colored with thiazine for 10 seconds, obtaining a blue coloration.

3.5. Protein Assay - bicinchoninic acid (BCA) assay

To determine the total concentration of protein of the semen samples, it was performed the bicinchoninic acid (BCA) assay, using a kit from Pierce and following the instructions of the manufacturer. This method is based in the reduction of Cu^{+2} to Cu^{+}, which is promoted by proteins in an alkaline medium (the biuret reaction); then, colorimetric detection of Cu+ cation is performed using a unique reagent containing bicinchoninic acid. The chelation of two molecules of BCA with one Cu^{+} ion is responsible for the formation of a purple-colored reaction product, which exhibits a strong absorbance at 562nm that is linear with increasing protein concentrations over a working range of 20 to 2000 µg/mL. This assay is not an end-point method because the final color continues to develop. However, following incubation, the rate of color development decreases, and that situation allows that a large number of samples can be assayed together.

Table 4- Preparation of diluted bovine serum albumin (BSA) standards to obtain the standard curve used in BCA protein assay method.

Standard	BSA (µL)	Protein mass (µg)	1% SDS (µL)	W.R. (µL)
P0	0	0	25	200
P1	0.5	1	24.5	200
P2	1	2	24	200
P3	2.5	5	22.5	200
P4	5	10	20	200
P5	10	20	15	200

BSA: Bovine Serum Albumin; SDS: Sodium Dodecyl Sulfate; W.R.: Working Reagent.

The samples were prepared to be assayed with 3 µL plus 22 µL of 1% SDS. To determine the total protein concentration of each sample we prepared the standard protein concentrations (Table 4). The stock solution of bovine serum albumin (BSA) used had a concentration of 2 mg/mL. The working reagent (W.R.) solution was prepared by mixing reagent A with reagent B, in a proportion of 50:1. Samples and standards were loaded (25 µL) in the 96-well microplate. Then, 200 µL of W.R. was added to each well and the microplate was incubated during 30 minutes at a temperature of 37°C. The microplate was then allowed to cool to room temperature during 5 minutes and the absorbance was measured at 562 nm in a microplate reader (TECAN, Genius, Männedorf, Switzerland). Using the standard protein concentrations and their subsequent absorbance, we obtained a standard curve that was used to determine the protein concentration of each sample.

3.6. Total Antioxidant Status

The concentration of antioxidants in sperm cells was measured using a commercial test for total antioxidant status (TAS) determination (Randox Laboratories, Crumlin, Northern Ireland). This test is based on the incubation of ABTS [2,2-azino-di-(3-ethylbenzthiazoiine sulphonate)] with a peroxidase (metmyoglobin) and H_2O_2; this incubation is responsible for the production of the radical cation $ABTS^+$, which has a relatively stable blue-green color, measured at 600 nm. The sperm cells contain antioxidants, and the addition of these cells to the reaction mixture causes suppression of the color formation; this suppression is proportional to the antioxidant capacity of the added sample.

This test was based in a kit composed by four reagents, which needed to be prepared to initiate the experiment (table 6). The buffer, phosphate buffered saline (PBS), is actually ready

for use, and it does not needed to be prepared. The chromogen (metmyoglobin) was presented in a solid state, so it was needed to add 10 mL of buffer to one vial of chromogen. The substrate, H_2O_2, needs to be diluted in buffer (1 mL of substrate R3 in 1,5 mL of the buffer). The standard was also presented in a solid state, so we reconstituted one vial of this reagent with 1 mL of ddH_2O.

Table 5 – Overview of the reagents of the TAS assay. In this table is presented the composition of the reagents, their methods of preparation and their stability after preparation.

Reagents			Preparation	Stability
R1	Buffer	Phosphate Buffered Saline (PBS)	Ready for use	To expiry date at +2 to +8ºC
R2	Chromogen	Metmyoglobin / ABTS®	Reconstitute one vial of chromogen R2 with 10 mL of Buffer R1	2 days at 2-8°C or 8 hours at 15-25°C
R3	Substrate	Hydrogen Peroxide	Dilute 1 mL of substrate R3 with 1.5 mL Buffer R1	24 hours at 2-8°C
CAL	Standard	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid	Reconstitute one vial of Standard with 1 mL of double deionized water	2 days at 2-8°C or 1 month at -20°C

In this experiment, first, ddH_2O or standard or sample were mixed with the chromogen (R2). Then, the mixture was brought to +37°C and after few minutes, the initial absorbance (A1) was read at 600 nm. Finally, substrate (R3) was added for exactly 3 minutes and the final absorbance (A2) was recorded. After these steps, the calculations were performed as following:

Calculation

$$A2 - A1 = \Delta A \text{ of sample/standard /blank}$$

$$\text{Factor} = \frac{\text{conc of standard}}{(\Delta A \text{ blank} - \Delta A \text{ standard})}$$

$$\text{mmol/l} = \text{Factor} \times (\Delta A \text{ blank} - \Delta A \text{ sample})$$

The reference range for human plasma blood is 1.30-1.77 mmol/L, according to manufacturer. However, the manufacturer does not had reference values for sperm cells, and the previous

studies performed in this area only analyzed the seminal plasma, with a range from 1,7 to 2,3 mmol/L.

3.6.1. Original Protocol

This assay had a protocol that was given by the company (Table 6) in which we must add 20 µl of the sample or standard or blank, in 1 mL of the chromogen and 200 µL of the substrate. Then, we used a cuvette to measure the mixture reaction at 600 nm in a spectrophotometer.

Table 6- Original protocol from the company.

Reagent / Samples	Blank	Standard	Sample
ddH_2O	20 µL		
Standard		20 µL	
Sample			20 µL
Chromogen (R2)	1 mL		
Substrate (R3)	200 µL		

3.6.2. Optimization of the protocol

However, in our laboratory we made a few alterations in the protocol (Table 7). The modified protocol was based in the total protein concentration and the cuvette method was modified to a microplate method with the subsequent scaling down of the volumes. All the samples had a protein concentration of 15 µg in a maximum volume of 5 µL. The samples with a higher concentration were diluted in 1% SDS. The amount of reagents used in this assay was also decreased. So, we used 5 µL of sample or ddH2O or standard, 200 µL of chromogen and 40 µL of substrate.

Table 7- Protocol modified to an equal protein concentration (15 µg).

Reagent / Samples	Blank	Standard	Sample
DDH_2O	5 µL		
Standard		5 µL	
Sample			5 µL
Chromogen (R2)	200 µL		
Substrate (R3)	40 µL		

3.7. Western Blotting

3.7.1. Preparation of SDS-PAGE gels:

In order to visualize the antioxidant proteins, SOD and GPx4, with molecular weights of approximately 18 and 20 kDa, respectively, we performed 15% SDS-PAGE big gels (Table 8). We used β-tubulin with a molecular weight of 50 kDa as a loading control.

Table 8- Composition of running and stacking gels for SDS-PAGE.

	Running gel (15%)	Stacking gel (3.5%)
H_2O	7.3 mL	6.6 mL
Acrylamide	15 mL	1.2 mL
4x LGB	7.5 mL	
5x UGB		2 mL
10% SDS		100 µL
10% APS	150 µL	100 µL
100% TEMED	15 µL	10 µL

APS: Ammonium Persulfate; LGB: Lower Gel Buffer; SDS: Sodium Dodecyl Sulfate; TEMED: Tetramethylethylenediamine; UGB: Upper Gel Buffer.

The preparation of the running gel was performed by adding sequentially the components that are described in Table 8. Ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were the last components to be added because they initiate the process of polymerization. The final solution is then pipetted down into the gel sandwich, leaving some space (approximately 4 centimeters) for the stacking gel. Water was then pipetted to cover the top of the running gel, allowing the gel to polymerize for 1 hour. The stacking gel was then prepared by adding the reagents that are present in the Table 8. The water was removed and the stacking gel was added to the sandwich gel. A comb was added in the top of the sandwich gel, and the solution was allowed to polymerize for 30 minutes.

3.7.2. Preparation and separation of the samples

A total amount of 50 µg of protein extract was prepared in 40 µL of total volume. Then, it was added one fourth of the volume of 4x LB. The samples were boiled at 100°C for 5 minutes and spin-down by centrifugation (1200 g, 1 minute). The combs were removed and the wells filled with running buffer. The samples were carefully applied into the wells and allowed to run at 90 mA until the bromophenol blue from the LB reached the bottom of the gel. A protein marker

(NZYColour Protein Marker II) was also applied for molecular weight comparison and to check the efficiency of the transfer.

3.7.3. Electroblotting

For electroblotting, the transfer cassette (Figure 6) was assembled as follows: 3MM blotter papers were cut to fit the transfer cassette and a nitrocellulose membrane was cut to fit the gel size. The SDS-PAGE gel was placed in the negative pole in contact with the nitrocellulose membrane that was turned to the positive pole, both between 3 MM blotter papers. Sponges were placed between the papers and the transfer cassette to compact.

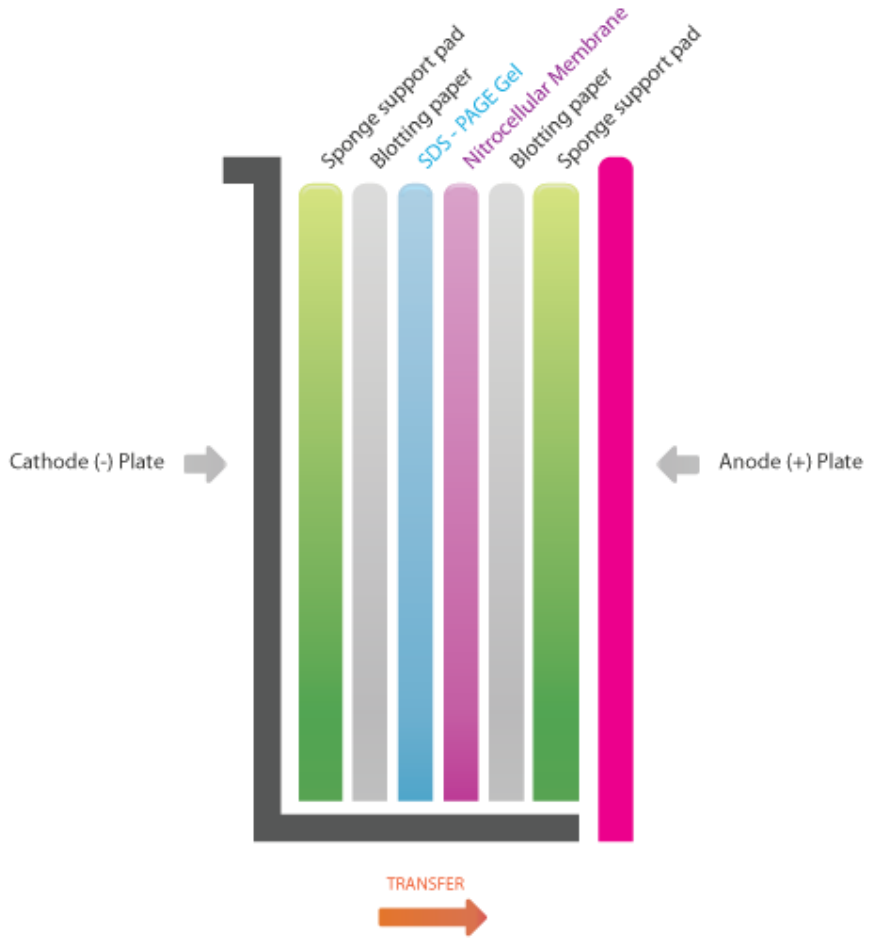

Figure 6- Preparation of the transfer cassette.

The gel was carefully removed from the electrophoresis tank and the stacking gel removed and discarded. The transfer sandwich was assembled in transfer buffer to avoid trapping air

bubbles. The cassette was placed in the transfer tank previously filled with transfer buffer. Transfer was allowed to proceed overnight (approximately 16 hours) at 200 mA. Afterwards, the transfer cassettes were disassembled; the membrane carefully removed and a Ponceau coloration was performed.

3.7.4. Ponceau Coloration

We performed a Ponceau coloration to visualize the proteins in the membrane. This step was useful to verify the presence of protein in each sample. After the transfer, we washed the membrane in 1x Tris Buffered Saline and Tween-20 (TBS-T). Then, we incubate the membrane with Ponceau on a shaker for 5 minutes. The membrane was then washed with distilled water until the proteins were well defined. To remove the coloration of the membrane, it was destained completely by repeated washing in distilled water.

3.7.5. Immunoblotting

The membrane was first hydrated with 1x TBS for 5 minutes. Then, the non-specific binding sites of the membrane were blocked in 1x TBS-T/5% low fat milk for 1 hour at room temperature with slow shaking. The membrane was then incubated for 2 hours with the primary antibodies anti-β-tubulin (clone AA2, Cat #05-661, Lot #2398882, monoclonal antibody), anti-SOD1 (clone 6F5, Cat# MABC864, Lot #VP1310030, monoclonal antibody) and anti-GPx4 (Cat #ABC269, Lot #VP1306033, polyclonal antibody). All antibodies were bought from Merck (Merck Millipore, Darmstadt, Germany) and were diluted 1:1000 in 1x TBS-T/5% low fat milk. After that, the membrane was washed with 1x TBS-T during 10 minutes, 15 minutes and 10 minutes, sequentially. Then, the membrane was incubated for 1 hour with the respective fluorescent secondary antibodies, 926-68071 IRDye 680RD Goat anti-Rabbit and 926-32210 IRDye 800CW Goat anti-Mouse(LI-COR BioSciences), diluted 1:5000 in 1x TBS-T/5% low fat milk: anti-mouse for β-tubulin and SOD1 and anti-rabbit for GPx4. Finally, the membrane was washed as before and scanned in LI-COR's Odyssey Infrared Imaging System (LI-COR Biosciences).

3.7.6. Stripping

A stripping was performed to allow the reprobing of the membranes. This step was performed using a fresh stripping solution. The membranes were incubated with this solution for 40 minutes at 55ºC, with a speed of 75 rpm. Then, the solution was removed and the membrane washed three times with 1x TBS-T for 15 minutes and two times with H_2O. The membranes were scanned in the Odyssey (LI-COR Biosciences). If the membranes appeared clear (meaning that at least the secondary antibody was removed), we further incubate them with secondary antibody, to confirm that both, primary and secondary antibodies were properly removed.

3.8. Carbonyl Group Determination

We performed a slot blot method followed by immunodetection for determination of the carbonyl groups.

3.8.1. Sample Derivatization

The samples were prepared following the procedure. In a microtube it was pipetted an amount of 30 µg of protein/50 µL of sample. The same volume (50 µL) of 12% SDS was added and the mixture spin-down for 5 seconds. Next, it was added 2 volumes (100 µL) of 20mM DNPH/10% trifluoroacetic acid (TFA). Then, the samples were incubated for 30 minutes in the dark. Finally, the solution was neutralized with 75 µL of 2M Tris/18% of mercaptoethanol.

3.8.2. Slot Blot

To perform the slot blot, the samples were diluted with 1x PBS to a concentration of 2 ηg/µL and 100 µL of each sample were applied in the slot blot (BioRad Portugal, Sintra, Portugal) and transferred to a nitrocellulose membrane. Then, the membrane was incubated in 10% methanol for activation. After this incubation, the membrane was washed in water and then in 1x TBS-T.

3.8.3. Immunodetection

The nitrocellulose membrane was blocked for 1 hour in 1x TBS-T/5% low fat milk. Next, the rabbit antibody anti-DNP, clone 9H8.1, MAB2223 (Merck KGaA, Darmstadt, Germany) with a concentration of 1:5000 was added and incubated for 1 hour. Then, the membrane was washed twice with 1x TBS-T for 15 minutes each. The membrane was then incubated with the anti-mouse secondary antibody (1:5000). Then, the membrane was washed two times with 1x TBS-T for 15 minutes each time and scanned using in Li-COR's Odyssey Infrared Imaging System (LI-COR BioSciences).

3.9. 3-Nitrotyrosine Determination

3.9.1. Sample Derivatization and Slot Blot

For 3-NT measurement, the spermatozoa samples were initially diluted in 1x TBS to obtain a final protein concentration of 1 ηg/μL. Then, a volume of 100 μL of these samples was slot-blotted into a nitrocellulose membrane.

As we did in the determination of carbonyl groups, the nitrocellulose membrane was then incubated with 10% methanol, to its permeabilization, and washed in water and 1x TBS-T.

3.9.2. Immunodetection

After these steps, it was added 5% milk in 1x T-TBS to block the nitrocellulose membrane. Then, we incubated the membrane with primary antibody anti-nitrotyrosine, cat# 06-284, lot# DAM1514077, rabbit polyclonal (Merck KGaA, Darmstadt, Germany), with a concentration of 1:1000. Then, it was washed two times with 1x T-TBS for 15 minutes each time. It was added the secondary antibody anti-rabbit 1:5000, for one hour. The membrane was again washed for two times with 1x T-TBS for 15 minutes and, finally, it was revealed in Li-cor's Odyssey Infrared Imaging System (LI-COR BioSciences).

3.10. Statistical Analysis

A longitudinal study approach was used to estimate the changes in sperm quality at three distinct time periods. All the statistical analysis was conducted using the SPSS 19.0 software. The individuals were followed, with few exceptions, before (TP1) and after (TP2 and TP3) the academic festivities weeks. In general, it was performed a descriptive statistic for each assay followed by a box-plot graph. In order to visualize the influence of the acute exposure and the alterations for each parameter, non-parametric tests were performed. For each assay, we performed Friedman test, to detect significant differences between TPs. To visualize the differences between each TP, two non-parametric paired tests were performed, according to the possibilities: Wilcoxon or Sign tests. It was also applied a Pearson and a Spearman correlation to evaluate the relation between each assay.

4. RESULTS

4.1. Project "*Para o Frasco 2010/2011"*

4.1.1. Lifestyle Alterations

To evaluate the alterations in the consumption of alcohol and nicotine, the questionnaires answered by the volunteers before (TP1) and after (TP2 and TP3) the academic festivities were analyzed. The sample was composed by a total of 47 volunteers, including 38 participants of "*Para o frasco 2010*" and 9 participants of "*Para o frasco 2011*".

4.1.1.1. Alcohol Consumption

First, it was analyzed the consumption of alcohol. To evaluate this alteration, we obtained the descriptive statistics of the answers of the 47 volunteers, which are described in Table 9. The results were calculated considering that each drink has a reference value of alcohol grams: a beer has approximately 15,84 g of alcohol, a wine glass has 16,8 g, a white alcoholic drink has 80 g and a shot has 16 g.

Table 9 – Descriptive statistics of the alcohol consumption. The minimum and the maximum consumes are described as well as the mean during time point 1 (TP1), time point 2 (TP2) and time point 3 (TP3). The results are presented in grams.

	TP1	TP2	TP3
N	47	47	36
Mean (g)	21,5929	155,5747	23,0991
Median (g)	15,8400	102,8571	14,3200
Standard Deviation (g)	20,81008	139,06184	34,70991
Minimum (g)	0,00	0,00	0,00
Maximum (g)	73,60	508,57	176,00

Observing Table 9, the mean of alcohol consumption at TP1 was approximately 21,59 grams of alcohol, while at TP2 the mean consumption was approximately 155,57 grams per day. Therefore, we verified that the consumption of alcohol per day increased more than 7 times between TP1 and TP2. At TP3, the alcohol consumption values decreased to basal levels (TP1).

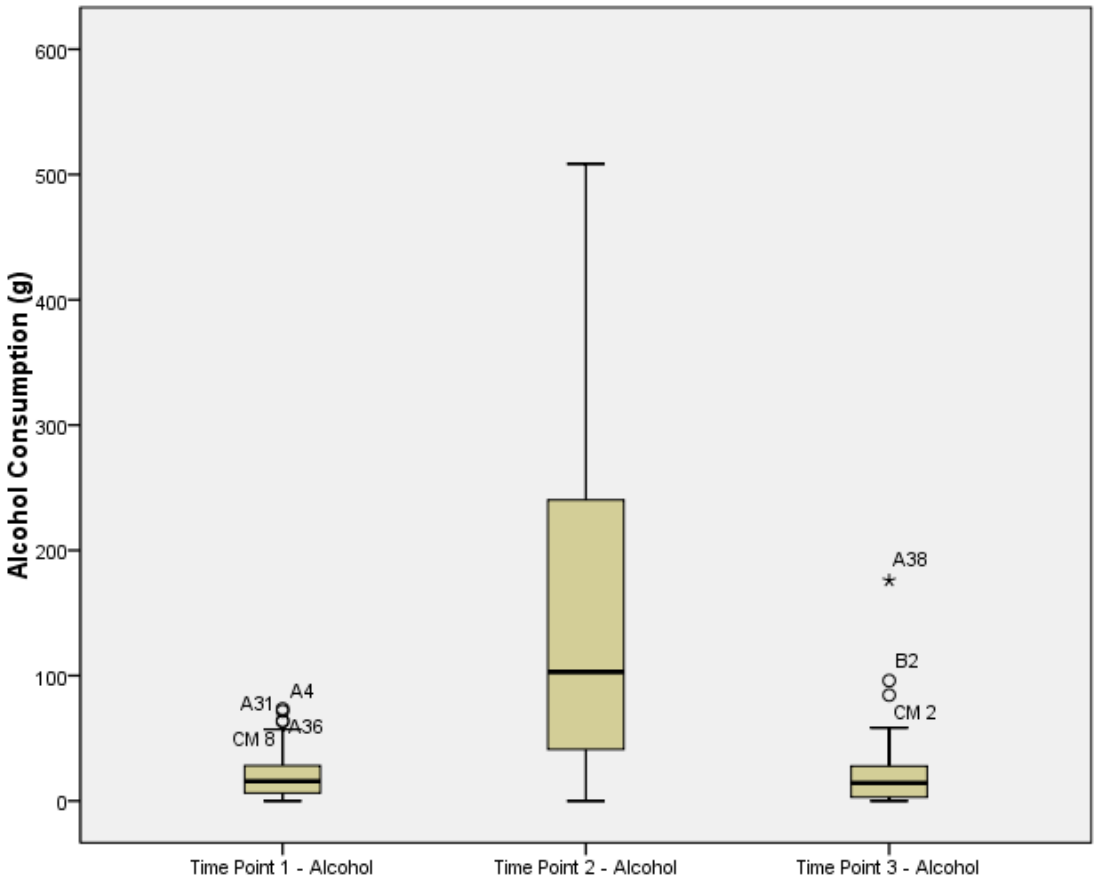

Figure 7 – Box plot of alcohol consumption before and during the academic festivities. It was observed an increase of alcohol consumption at TP2.

When analyzing the box-plot (Figure 7) of the three time points it was possible to verify, once again, that there was an increase of alcohol consumption at TP2. To evaluate the statistical significance between the TPs it was performed a Sign Test, that is a non-parametric test that compares two or more paired samples (Table 10). This test was used due the presence of assimetry (median is not centered in the box-plot) observed in Figure 7. In all the statistical tests of this study it was considered a significance level of 5% (α= 0,05). We also opted to perform non-parametric tests due to the reduced number of samples.

Table 10 – Sign test for alcohol consumption.

	TP2 vs TP1	TP3 vs TP1	TP3 vs TP2
Asymp. Sig. (2-tailed)	0,000	0,728	0,000

TP: Time Point

By performing the Sign Test we concluded that there was a significant increase in the alcohol consumption at TP2, because of the p value < 0,05 (p= 0,000). However, TP1 and TP3 showed a p value > 0,05, which means that the consumption of alcohol at these TPs has not showed a significant difference. However, when a multiple pair test, as the Sign Test, is performed, there is a chance of obtaining false-positive results. Therefore, to confirm the results obtained, it was performed Bonferroni correction (Table 11), which is an adjustment made to p values when several dependent statistical tests are being performed.

Table 11- Bonferroni correction for the Sign Test used to determine alcohol consumption.

	TP2 vs TP1	TP3 vs TP1	TP3 vs TP2
Asymp. Sig. (2-tailed)	0,000	1	0,000

TP: Time Point

As we can observe in Table 11, the p value at TP2 was < 0,05 (p= 0,000), which confirms the significant increase at this TP, obtained with the Sign Test.

4.1.1.2. Nicotine Consumption

After the analysis of alcohol consumption, we focused in the consumption of nicotine. To evaluate this alteration we first obtained the descriptive statistics for answers to the questionnaires, considering that each cigarette has 1,2 mg of nicotine. The results are presented in Table 12.

Table 12 - Descriptive statistics of the nicotine consumption. The minimum and the maximum consumes are described as well as the mean during time point 1 (TP1), time point 2 (TP2) and time point 3 (TP3). The results are presented in milligrams.

	TP 1	TP 2	TP3
N	47	47	36
Mean (mg)	1,9021	3,4213	1,2000
Median (mg)	0,0000	0,0000	0,0000
Standard Deviation (mg)	4,26163	5,65612	2,82519
Minimum (mg)	0,00	0,00	0,00
Maximum (mg)	18,00	20,40	12,00

We concluded that, on average, the consumption of nicotine increased almost 80% during the academic week.

Analyzing the box-plot (Figure 8), where is presented the median of each TP, it was possible to verify a slight increase at TP2. We could also observe that the volunteer CM4 had a decrease in the consumption of nicotine.

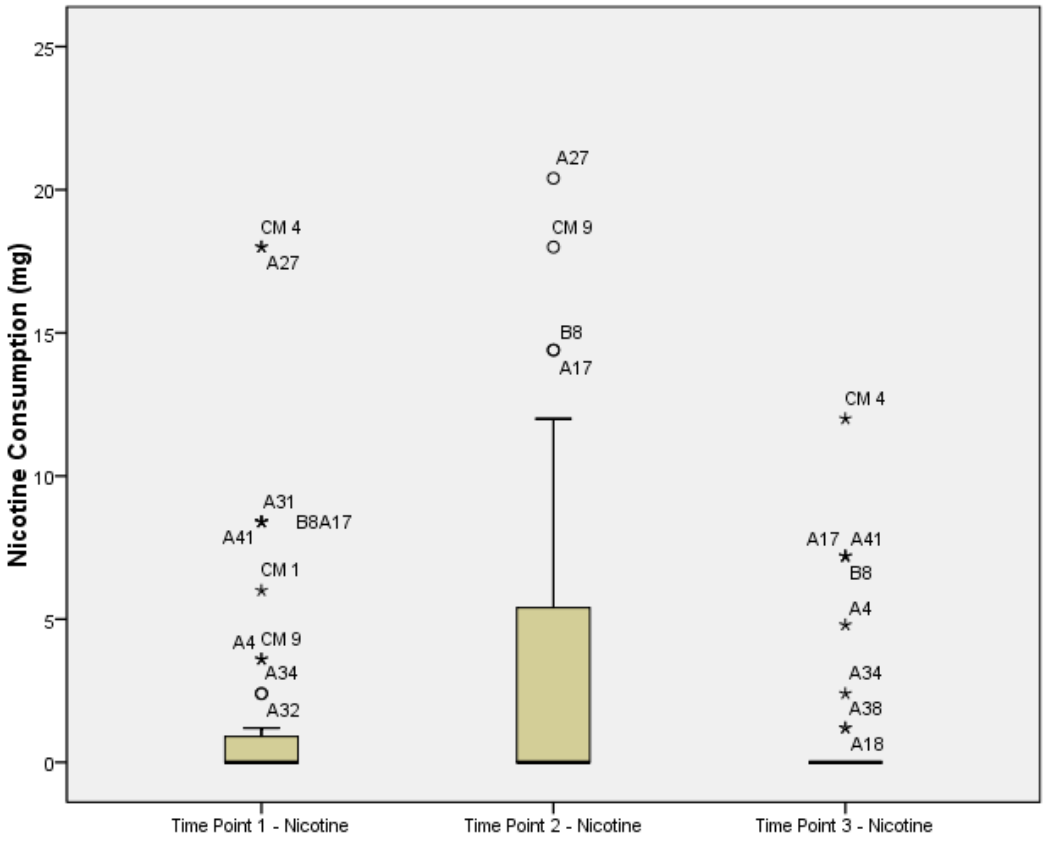

Figure 8 – Box plot of nicotine consumption before and during the academic week. It was verified an increase of nicotine consumption at TP2.

To confirm if there was a significant difference in the consumption of nicotine between the three TPs, it was performed a Sign Test (Table 13). This test showed that the nicotine consumption increase during the academic week was statistical significant, because p value< 0,05 (p value= 0,001). However, the differences verified between TP1 and TP3 were not statistical significant, because p value > 0,05 (p= 0,453).

Table 13 – Sign Test for nicotine consumption.

	TP2 vs TP1	TP3 vs TP1	TP3 vs TP2
Asymp. Sig. (2-tailed)	0,001	0,453	0,001

TP: Time Point

Then, it was performed Bonferroni correction (Table 14) that confirmed the results obtained in the Sign Test.

Table 14- Bonferroni correction for the Sign Test used to determine nicotine consumption.

	TP2 vs TP1	TP3 vs TP1	TP3 vs TP2
Asymp. Sig. (2-tailed)	0,003	1	0,003

TP: Time Point

4.1.2. Antioxidants

To study the antioxidant defenses of sperm cells it was performed two different assays: TAS, to evaluate the total antioxidant capacity of these cells and Western Blotting, to evaluate the expression levels of two specific antioxidant proteins: SOD and GPx4. To standardize the samples, it was used the amount of β-tubulin, performing a ratio SOD/β-tubulin or GPx4/β-tubulin, for each sample. The results were analyzed using statistical tests.

4.1.2.1. Total Antioxidant Status

The TAS assay was performed to evaluate the antioxidant capacity of the sperm cells. As observed in Table 15, to perform this test it was used a total of 48 samples, given by 18 different volunteers; 7 volunteers from "*Para o Frasco 2011*", and 11 volunteers from "*Para o Frasco 2010*". TAS is a spectrophotometric assay and the obtained values were analyzed in SPSS software. In Table 15 we can also observe that the mean as well as the median of TAS increased at TP2. To visualize these results it was performed a box plot, represented in Figure 9.

Table 15 – Descriptive statistics of the samples used to perform TAS tests, at the different time points.

	TP1	TP2	TP 3
N	16	18	14
Mean (mmol/L)	1,5964	1,7445	1,4693
Median (mmol/L)	1,785	1,895	1,725
Standard Deviation (mmol/L)	0,45303	0,39967	0,62932
Minimum (mmol/L)	0,61	0,68	0,27
Maximum (mmol/L)	2,12	2,23	2,03

TP: Time Point.

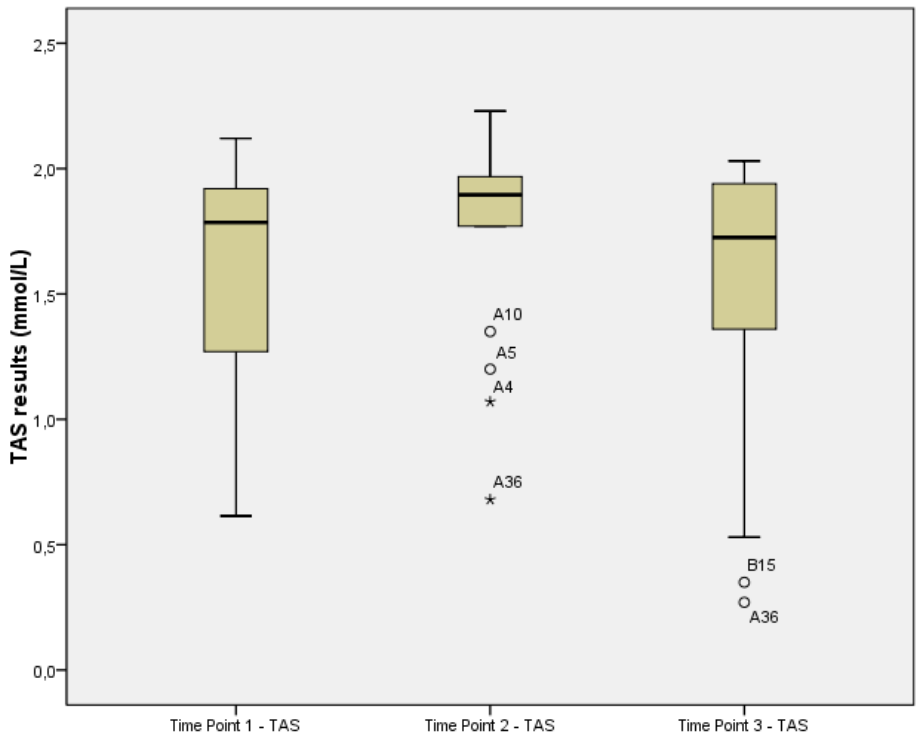

Figure 9 – TAS results at the three different time points.

We performed Friedman test (Table 16) to determine if the differences between the three time points were statistically significant. Friedman Test is a non-parametric statistical test that is used to detect differences between multiple paired samples; however this test is different of Wilcoxon or sign tests because it does not detect where are localized the differences.

Table 16 – Friedman test for TAS determination.

Friedman Test	
N	12
Asymp. Sig.	0,558

As we can observe in Table 16, there was no significance detected (p= 0,558) in this test so we can conclude that there is no significant difference between TPs during the realization of this assay. For this reason it was not performed the Wilcoxon or Sign tests.

4.1.2.2. SOD Expression

Then, we measured the presence of SOD, an antioxidant protein, by Western Blot in a total of 84 samples. In this assay, as in the others, the number of analyzed samples was not coherent at the different TPs (Figure 10).

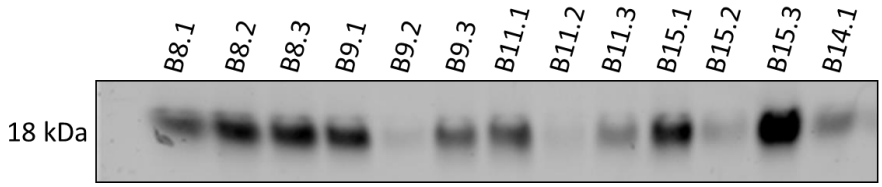

Figure 10- Representative blot of SOD expression detected by Western blot assay.

The obtained results were then analyzed in SPSS software. We first performed a descriptive statistic, where the values of mean, median and standard deviation and others parameters were analyzed. These statistics are presented in Table 17.

Table 17 – Descriptive statistics of SOD expression at the different time points.

	TP1	TP2	TP3
N	39	20	25
Mean	1,2376	1,5881	14,7356
Median	0,7176	0,7254	6,8656
Standard Deviation	1,34201	3,42109	19,15576
Minimum	0,30	0,15	0,51
Maximum	7,89	15,95	81,42

TP: Time Point.

Observing Table 17 it is possible to visualize an increase in the presence of SOD over the TPs, however the highest increase of SOD occurred between TP2 and TP3. To complement the results obtained in Table 17 it was performed a box plot for these samples, which is presented in Figure 11.

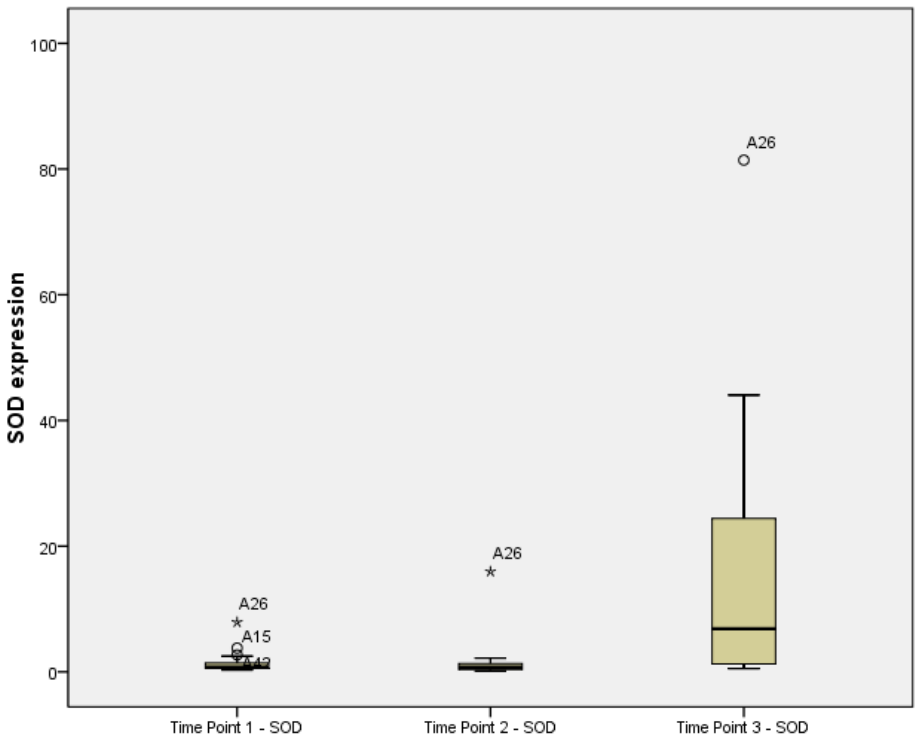

Figure 11 – Box-plot of the normalized samples of SOD expression.

Next, we performed a Friedman Test (table 18), to detect differences between the samples of the same individuals, obtaining a p value < 0,05 (p= 0,009). With this result we concluded that there is a significant alteration between the three time points analyzed.

Table 18 – Friedman Test for the expression of SOD.

Friedman Test	
N	13
Asymp. Sig.	0,009

To detect the time points that are significantly different between them, showed by Friedman Test, we needed to perform a multiple comparison using the sign test (Table 19). This test showed the presence of statistical significance at TP3, when comparing with the other TPs. These results were then confirmed by the Bonferroni correction (Table 20).

Table 19 – Sign Test results for the expression of SOD.

	TP2 vs TP1	TP3 vs TP1	TP3 vs TP2
Asymp. Sig. (2-tailed)	0,804	0,000	0,007

TP: Time Point

Table 20 - Bonferroni correction for the sign test used to determine the expression of SOD.

	TP2 vs TP1	TP3 vs TP1	TP3 vs TP2
Asymp. Sig. (2-tailed)	1	0,000	0,021

TP: Time Point

4.1.2.3. GPx4 Expression

Then, we proceeded to the evaluation of GPx4 expression, by Western Blot in a total of 71 samples (36 from TP1, 20 from TP2 and 25 from TP3) (Figure 102).

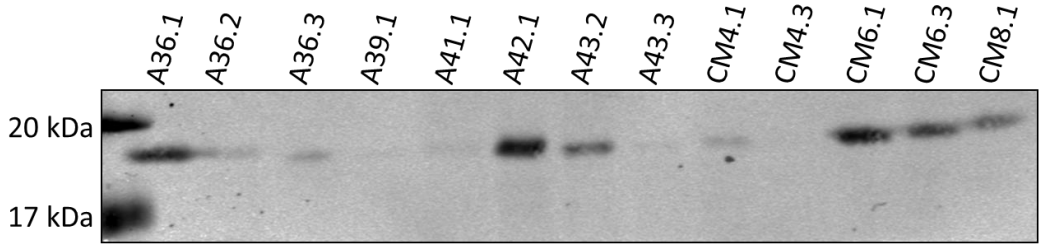

Figure 12- Representative blot of GPx4 expression detected by Western blot assay.

The results were then statistically analyzed. We first performed a descriptive statistic, where the values of mean, median and standard deviation and others parameters were analyzed. These statistics are presented in Table 21.

Table 21 – Descriptive statistics of GPx4 expression at the different time points.

	TP1	TP2	TP3
N	36	20	25
Mean	0.4212	0.3050	1.7304
Median	0.3129	0.1926	0.5133
Standard Deviation	0.66538	0.36604	4.07592
Minimum	0.00	0.01	0.00
Maximum	4.02	1.63	19.68

TP: Time Point.

Then, we performed a box-plot (Figure 13), where it was observed the distribution of the samples results.

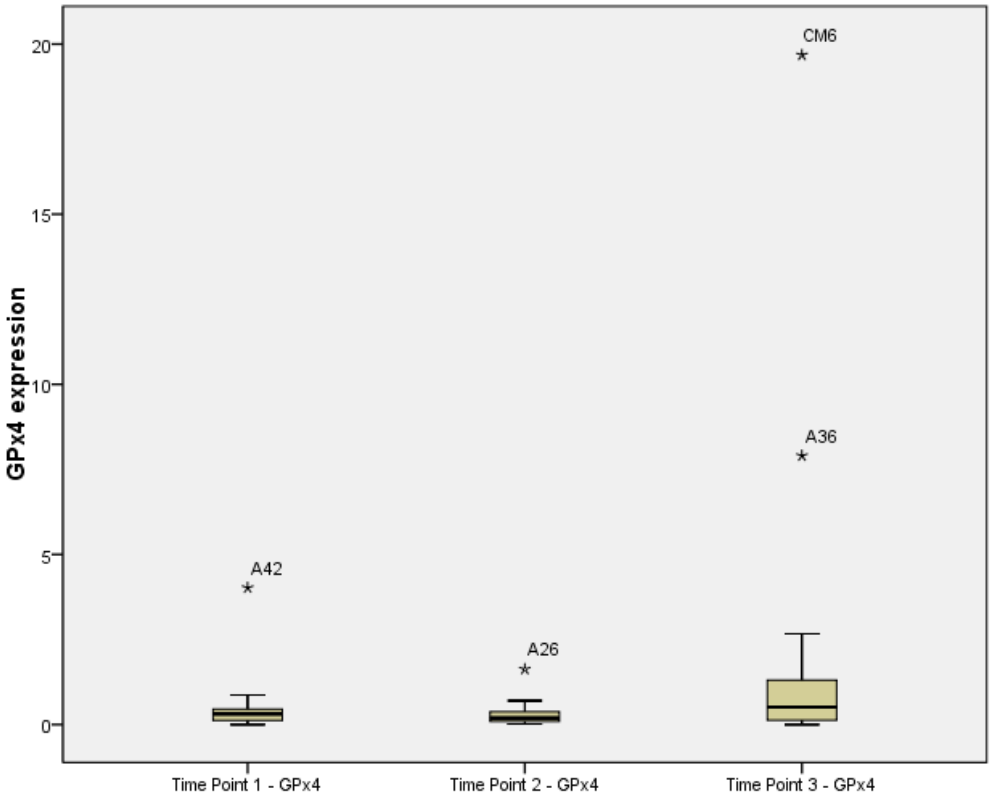

Figure 13 – Box-plot of GPx4 expression at three different time points.

To evaluate the presence of differences at the different time points for each volunteer we performed Friedman Test, but as we can observe in Table 22, the p value > 0,05 (p= 0,558), which means that it was not observed a significant statistical difference between the TPs.

Table 22 – Friedman test to evaluate GPx4 expression.

Friedman Test	
N	12
Asymp. Sig.	0,558

4.1.3. Oxidative Stress

We evaluated the effect of the oxidative stress in the sperm cells, more concretely in the protein oxidation status. This evaluation was performed by detecting the presence of 3-NT.

4.1.3.1. 3-Nitrotyrosine Determination

We first evaluated the presence of 3-NT in the samples by the slot-blot technique. The obtained results are represented in Figure 14. A total of 99 samples (39 from TP1, 29 from TP2 and 31 from TP3) were analyzed, which were standardized using the results of Ponceau coloration, performing a ratio 3-NT/Ponceau.

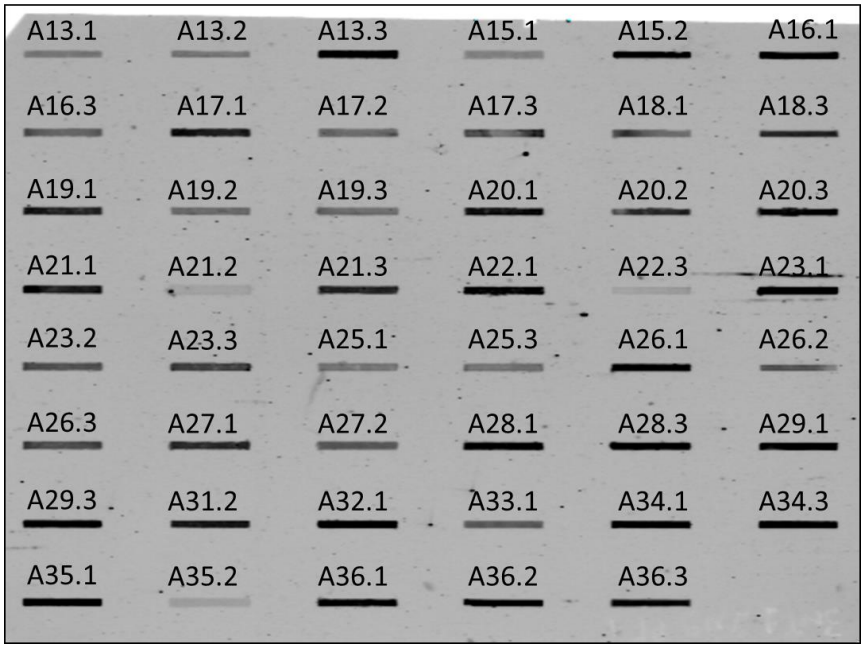

Figure 14- Results obtained with 3-NT determination by slot-blot.

The results were then analyzed in the SPSS software. First, we obtained a descriptive statistic of 3-NT (Table 23).

Table 23 – Descriptive statistics of 3-nitrotyrosine determination at the different time points.

	TP1	TP2	TP3
N	39	29	31
Mean	1.4296	1.8718	0.9401
Median	1.2234	1.7413	0.8904
Standard Deviation	0.78763	1.03542	0.40121
Minimum	0.42	0.39	0.27
Maximum	4.11	5.24	1.63

TP: Time Point.

By observing table 23, we can conclude that the mean of the presence of 3-NT has slight increased in the TP2, compared to TP1. However, it was also seen a decrease of the expression of this protein in the TP3, compared to TP1 and TP2. A box-plot of the samples was performed to visualize the median and the distribution of the samples (Figure 15).

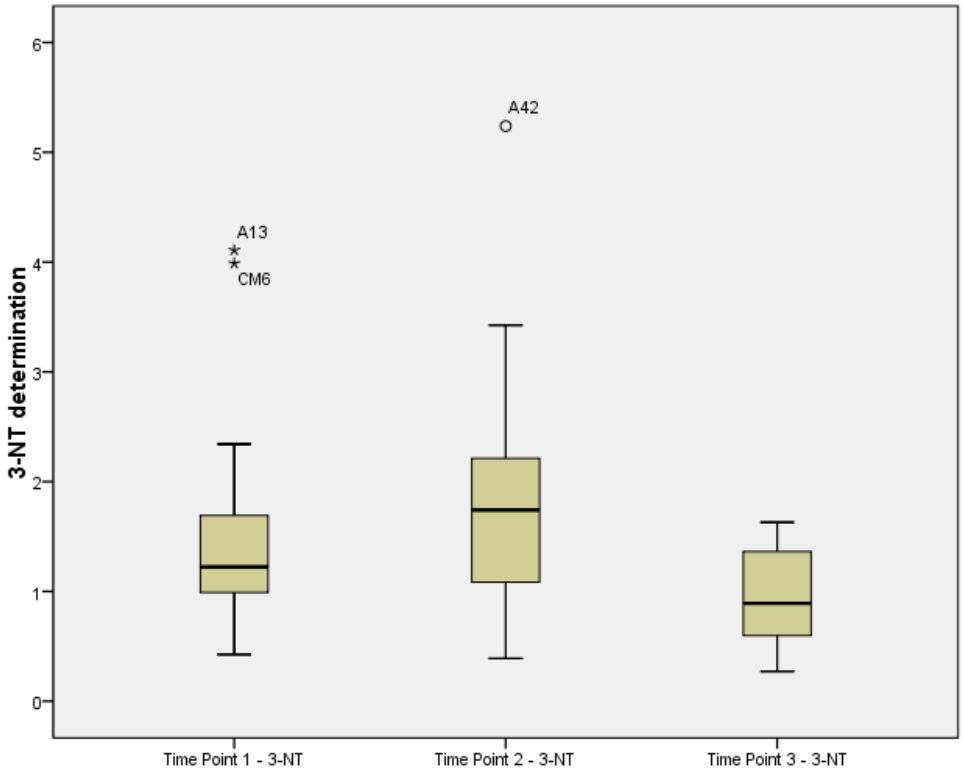

Figure 15 – Box plot of the 3-NT groups at the three different time points.

To verify the relation between the different TPs it was performed a Friedman Test that is presented in Table 24 With this test we can conclude that the samples are significantly different between the different TPs, because the p value < 0,05 (p= 0,001).

Table 24 – Friedman Test of 3-NT.

Friedman Test	
N	16
Asymp. Sig.	0,001

To detect the time points that are significantly different between them, we performed a non-parametric test that allowed us to compare repeated measurements of the same sample. As the box-plot (Figure 15) of the 3-NT was symmetric for the three boxes, we decided to perform the Wilcoxon test (Table 24).

Table 25 – Wilcoxon Test of 3-NT. TP: Time Point

	TP2 vs TP1	TP3 vs TP1	TP3 vs TP2
Asymp. Sig. (2-tailed)	0,019	0,000	0,002

The Wilcoxon test showed that there was a significant difference between all the TPs. These results were then verified by Bonferroni correction (Table 26), which showed a difference in the correlation of TP1 and TP2, with a p value > 0,05. However, as this test demands a very reduced p value obtained in Wilcoxon test, associated with our decrease number of samples, we will consider the result obtained by Wilcoxon Test in this case.

Table 26- Bonferroni correction for the Wilcoxon test used to determinate the presence of 3-NT.

	TP2 vs TP1	TP3 vs TP1	TP3 vs TP2
Asymp. Sig. (2-tailed)	0,057	0,000	0,006

TP: Time Point

4.1.4. Correlations between tests

After the realization of the tests previously described, we performed a Pearson correlation coefficient (Table 27) to measure the dependence of the tests.

Table 27 – Pearson correlation between the tests performed.

		SOD	GPx4	TAS	3-NT
SOD	Pearson Correlation	1	,514**	-,135	-,352*
	Sig. (2-tailed)		0,000	0,529	0,018
	N	53	43	24	45
GPx4	Pearson Correlation	,514**	1	-,122	-,268
	Sig. (2-tailed)	0,000		0,589	0,076
	N	43	49	22	45
TAS	Pearson Correlation	-,135	-,122	1	,147
	Sig. (2-tailed)	0,529	0,589		0,430
	N	24	22	42	31
3-NT	Pearson Correlation	-,352*	-,268	,147	1
	Sig. (2-tailed)	0,018	0,076	0,430	
	N	45	45	31	70

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

3-NT: 3-Nitrotyrosine; GPx4: Glutathione Peroxidase 4; SOD: Superoxide Dismutase; TAS: Total Antioxidant Status.

Observing Table 27, we can conclude that there is a correlation between some of the assays performed: there is a positive correlation between SOD and GPx4 (p= 0,000) and a negative correlation between SOD and 3-NT (p=0,018).

4.2. Relation between seminal quality and oxidative balance

For this study, a total of 32 volunteers samples were used from the project for *APU*. It was performed a spermogram (or semen analysis) to all the semen samples from these volunteers. However, the other assays (TAS, SOD, GPx4 and 3-NT) were not performed in all the samples due to the lack of samples or because the samples were too diluted (especially to perform TAS assay).

After performing the spermogram, TAS assay, Western Blot (SOD and GPx4) and Slot-blot (3-NT and CG), we statistically analyzed the data using the SPSS software. Our objective was to analyze the possible relations between the seminal parameters evaluated in spermogram with the alterations in antioxidants and oxidized proteins levels. To analyze these relations it was

performed a Spearman correlation (Table 28). This test was performed due to the presence of ordinal values.

Table 28 – Spearman correlation of *APU* samples.

Correlations

			TAS	SOD	GPx4	3-NT	CG
	Viscosity	Correlation Coefficient	-0,260	-0,514	-0,212	-0,040	0,220
		Sig. (2-tailed)	0,199	0,035	0,413	0,848	0,313
		N	26	17	17	25	23
	Volume	Correlation Coefficient	-,038	-,664	-,436	,011	,379
		Sig. (2-tailed)	0,852	0,004	0,080	0,959	0,074
		N	26	17	17	25	23
	Concentration	Correlation Coefficient	-0,380	0,039	0,466	-0,011	0,309
		Sig. (2-tailed)	0,056	0,881	0,059	0,957	0,152
		N	26	17	17	25	23
	Number of	Correlation Coefficient	-0,387	-0,077	0,375	-0,023	0,407
		Sig. (2-tailed)	0,051	0,768	0,138	0,913	0,054
		N	26	17	17	25	23
	Motility	Correlation Coefficient	0,018	0,147	0,277	-0,349	-0,041
		Sig. (2-tailed)	0,934	0,588	0,299	0,095	0,855
		N	23	16	16	24	22
	Progressive	Correlation Coefficient	0,005	0,186	0,447	-0,273	0,067
		Sig. (2-tailed)	0,983	0,489	0,083	0,197	0,766
		N	23	16	16	24	22
	Non-sive motility	Correlation Coefficient	0,019	-0,130	-0,610	-0,057	-0,186
		Sig. (2-tailed)	0,933	0,632	0,012	0,792	0,408
		N	23	16	16	24	22
	Non-motility	Correlation Coefficient	-0,030	-0,094	-0,288	0,312	0,046
		Sig. (2-tailed)	0,893	0,730	0,279	0,138	0,840
		N	23	16	16	24	22
	Normal logy	Correlation Coefficient	-0,073	0,092	0,442	0,146	-0,095

	Sig. (2-tailed)	0,740	0,735	0,086	0,496	0,673
	N	23	16	16	24	22
Head Defects	Correlation Coefficient	0,010	-0,094	-0,244	-0,198	-0,008
	Sig. (2-tailed)	0,964	0,728	0,363	0,353	0,972
	N	23	16	16	24	22
MiddlePiece	Correlation Coefficient	-0,174	-0,060	-0,228	-0,171	-0,090
	Sig. (2-tailed)	0,426	0,826	0,396	0,423	0,691
	N	23	16	16	24	22
Tail Defects	Correlation Coefficient	0,217	0,508	-0,021	0,099	-0,032
	Sig. (2-tailed)	0,320	0,044	0,939	0,646	0,888
	N	23	16	16	24	22
TAS	Correlation Coefficient	1,000	0,411	-0,242	-0,112	-0,266
	Sig. (2-tailed)	.	0,128	0,386	0,649	0,285
	N	26	15	15	19	18
SOD	Correlation Coefficient	0,411	1,000	0,381	0,389	-0,702
	Sig. (2-tailed)	0,128	.	0,131	0,151	0,005
	N	15	17	17	15	14
GPx4	Correlation Coefficient	-0,242	0,381	1,000	0,375	-0,435
	Sig. (2-tailed)	0,386	0,131	.	0,168	0,120
	N	15	17	17	15	14
3-NT	Correlation Coefficient	-0,112	0,389	0,375	1,000	-0,101
	Sig. (2-tailed)	0,649	0,151	0,168	.	0,647
	N	19	15	15	25	23
CG	Correlation Coefficient	-0,266	-0,702	-0,435	-0,101	1,000
	Sig. (2-tailed)	0,285	0,005	0,120	0,647	.
	N	18	14	14	23	23

3-NT: 3-Nitrotyrosine; CG: Carbonyl Groups; GPx4: Glutathione Peroxidase 4; SOD: Superoxide Dismutase; TAS: Total Antioxidant Status.

Spearman correlation demonstrated some relations between seminal parameters and the proteins analyzed (Table 28). We concluded that SOD is negatively correlated with viscosity as well as volume, and carbonyl groups presence. This protein also showed a positive correlation

with the presence of tail defects in sperm cells. In turn, GPx4 showed a negative correlation with the presence of non-progressive motile spermatozoa.

5. DISCUSSION

5.1. "*Para o Frasco*" Studies

Oxidative stress has been studied as one of the causes of idiopathic infertility. In the reproductive system it has been described that some lifestyle factors, such as alcohol and nicotine consumption, may increase the levels of ROS (Lavranos et al., 2012; Tremellen, 2008). This increase may damage the lipids, DNA and proteins of the spermatozoon leading to a loss of function and eventually to cell death (Tvrdá et al., 2011; Agarwal et al., 2008; Makker et al., 2009). However, sperm cells have mechanisms of defense to prevent the action of ROS, the antioxidant proteins. These molecules counteract the effects of ROS, such as $O_2^{\bullet-}$ and H_2O_2, decreasing their levels in the cells.

"*Para o Frasco*" was a study that evaluated the influence of acute lifestyle changes, such as the consumption of alcohol and tobacco, during the academic festivities, in the male fertility. The sperm samples were given by young male volunteers, in reproductive age, at University of Aveiro, during three defined different moments: the first moment (time point 1 – TP1) was one week before the academic week, the second moment (time point 2 – TP2) was one week after the academic festivities and the third moment (time point 3 – TP3) was around three months after this festivity. The volunteers answered to a questionnaire in each time point, when they delivered their samples. To perform this study, a total of 47 volunteers were included, 9 volunteers were from "*Para o Frasco*" 2011 and 38 volunteers were from "*Para o Frasco*" 2010; however, 11 of these volunteers did not participate at TP3. During the analysis of the questionnaires it was concluded that the consumption of alcohol during the academic week increased significantly between TP1 and TP2 ($p= 0,000$), to values higher than 7 times; in turn, at TP3 the consumption values returned to basal levels. The consumption of nicotine has also increased significantly between TP1 and TP2 ($p=0,001$), to values 0,8 times higher. However, it must be referred that it is very challenging to quantify the consumption of alcohol and nicotine because the attributed values were calculated based on the answers of the questionnaires. Furthermore, we must refer that the alterations observed during this study can not only be attributed to the alterations in the consumption of these substances, since there are other factors that may interfere in the quality of the semen that we cannot quantify accurately, such as the alterations in diet and in circadian rhythm, among others. Indeed, an acute life style alteration occurs during the academic festivities week that impacts sperm quality (Ferreira et al., 2012).

To directly and indirectly evaluate the OS in sperm cells, we performed three different types of assays. First, we evaluated the total antioxidant capacity of the sperm cells, by performing the TAS assay. Then, we proceeded to evaluate the presence of specific antioxidant proteins, namely SOD and GPx4. At last, we evaluated the protein oxidation, measuring 3-NT levels. Samples from "*Para o Frasco*" 2010 and 2011 were used, resulting in a total of 86 samples analyzed. We did not always have 3 samples from each volunteer since not all gave samples in all 3 TP and additionally, since the samples were from previous studies, some of them had already been fully used. In turn, it was also not possible to analyze all the samples in all the assays performed (spectrophotometric, immunoblotting and slot blotting), due to two different factors: first, as the samples were from previous studies we do not had the necessary amount to perform the assays; second, some samples were too diluted, being impossible to use the desired volume with the needed concentration.

We started the study of the oxidative balance in sperm cells by performing the TAS assay, an assay used for the quantitative determination of the antioxidant capacity of the sperm cells. This assay is based in the incubation of ABTS with a peroxidase and H_2O_2, producing the radical cation $ABTS^{+}$, which has a blue-green color and is measured at 600 nm. The antioxidants of the added samples caused suppression of the color formation. So, for higher concentrations of antioxidants, less color will be produced and thus, the obtained value will be lower. We concluded that between TP1 and TP2, the obtained value for this assay slightly increased, which represents a decrease of the antioxidant capacity of the sperm cells. However, at TP3 the value decreased to a similar value of TP1 meaning that in these samples the antioxidant capacity has been restored. The ROS that is involved in the reaction in this assay is H_2O_2, so this test only evaluates the antioxidants that interact with this molecule. As it was previously referred, GPx4 is one of the antioxidant proteins that interact with this ROS, converting it into H_2O. So indirectly, this assay also evaluated the levels of GPx4 as well as other antioxidants not evaluated in this work, such as CAT. The results for the different TPs were not statistically significant however the reduced number of analyzed samples might explain this situation.

After evaluating the antioxidant capacity of the sperm cells, we performed an evaluation of the expression levels of specific antioxidant proteins, concretely GPx4 and SOD (Cu/Zn isoform). The results for each sample were standardized with β-tubulin, a widely used loading control, through the following ratio: antioxidant protein (SOD or GPx4)/ β-tubulin.

SOD is an antioxidant protein that is responsible for the conversion of $O_2^{\bullet -}$ into H_2O_2 and O_2 (Alvarez, et al., 1987). The specific SOD isoform evaluated was the CuZnSOD that is localized in

the cytoplasm of the sperm cells. This isoform is highly expressed in spermatogonia, being less expressed in spermatocytes, spermatids and spermatozoa (Nonogaki et al., 1992; Celino et al., 2011); however, the expression of this protein in these cells after acute stimulation of OS has never been evaluated. In addition to spermatogonia, ductus deferens and prostate cells also have high levels of expression of this SOD isoform (Nonogaki et al., 1992; Celino et al., 2011). By Western blot we have confirmed that SOD increases after TP1, which means that the acute lifestyle changes lead to an alteration of the levels of this protein. Nevertheless, SOD is much more expressed at TP3 being the increase, when compared to TP1, significantly different (p= 0,000). Therefore, the small increase of SOD expression by sperm cells at TP2 can be explained by the acquisition of this protein from the seminal fluid. At TP3, we analyzed the spermatozoa that were germ cells during the academic week. Thus, increased levels of SOD in these spermatozoa can be explained by an exponential increase of OS in the testis, which lead to a higher expression of CuZnSOD in spermatogonia, but thereafter is maintained in the later steps of spermatogenesis. Another explanation is also possible: at the academic week, ROS levels are significantly increased and SOD produced in the testis is not sufficient to eliminate ROS, so spermatozoa have to acquire the antioxidants present in the seminal fluid.

Glutathione Peroxidase 4, as previously referred, is an antioxidant enzyme that leads to the reduction of H_2O_2 into H_2O, and it is found essentially in testis and in the nucleus and mitochondria of mature sperm cells (Chabory et al., 2010). By evaluating the presence of this antioxidant we observed a decrease of its levels during TP2, but also an increase during TP3, when comparing with TP1. However, these alterations were not statistically significant. These results are consistent with the results of TAS assay, but no statistical correlation was found between these two tests. In previous studies, GPx4 was shown to exist as a soluble peroxidase (enzymatically active) in spermatids, but in mature spermatozoa the mitochondrial isoform is inactive, having a structural role (Chabory et al., 2010; Ursini et al., 1999). The low decrease in GPx4 levels in TP2 can be explained by spermatozoa presence already in the epididymis, which only expresses an isoform of GPx4 (cytosolic GPx4) that does not exists in mature spermatozoa (Chabory et al., 2010; Noblanc et al., 2011). At TP3, spermatozoa that were germ cells during the academic week were analyzed. As GPx4 is highly present in spermatids, their levels during spermatogenesis will be increased, leading to an increase in expression of this protein when the sperm cells are ejaculated.

To evaluate the damage of sperm cells caused by ROS we checked for the presence of 3-NT groups. These molecules are products of the tyrosine nitration, mediated by peroxynitrite ($ONOO^-$) and it is a common marker of cell damage and of OS. Peroxynitrite is a molecule that

is produced by the reaction of $O_2^{\bullet-}$ and NO (Pacher, Beckman, & Liaudet, 2007). An increase of 3-NT was observed in TP2 (p= 0,019); however a marked decrease occurred in TP3 (p= 0,000). Protein modifications by tyrosine nitration potentially result in alteration, loss or gain of function. 3-NT was measured by performing a slot-blot, and the results were negatively correlated with SOD. Alcohol and nicotine are responsible for increasing ROS levels in spermatozoa, seminal fluid and testis (Lavranos et al., 2012; Tremellen, 2008). So, if the concentration of $O_2^{\bullet-}$ and NO is increased, $ONOO^-$ levels will increase too, leading to an accentuated presence of tyrosine nitration.

After the performance and individual analysis of these assays, we analyzed the possible relations between the studied proteins. First, to better understand the correlations, Figure 16 should be analysed. The $O_2^{\bullet-}$ is converted to H_2O_2, by the action of SOD; then, this molecule is reduced to H_2O by GPx4, a protein that complements SOD action, eliminating ROS. However, $O_2^{\bullet-}$ can also interact with NO to produce peroxynitrite ($ONOO^-$) (Pacher et al., 2007). This molecule is a powerful antioxidant that will cause tyrosine nitration and consequent formation of 3-NT.

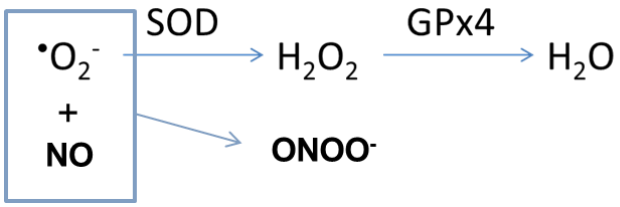

Figure 16 – Scheme representing the interaction between ROS and antioxidant proteins.

In Table 29 all the correlations verified with the Pearson test are represented. Superoxide dismutase is highly correlated (p=0,000) with GPx4; that means that when the levels of SOD increase there is also an increase in the levels of GPx4. Observing Figure 16, this situation is easily explained. SOD converts $O_2^{\bullet-}$ in H_2O_2, which is also a ROS that damages the sperm cells, and the sperm cells will improve the production of GPx4 (or other antioxidants that were not evaluated) to convert H_2O_2 in water molecules (H_2O). In resume, GPx4 completes the action of SOD.

Table 29 – Overview of the correlated assays from "*Para o Frasco*" studies.

Assay	Relation	Type of correlation	Significance
SOD	GPx4	+	0,000
	3-NT	-	0,018

3-NT: 3-Nitrotyrosine; GPx4: Glutathione Peroxidase 4; SOD: Superoxide Dismutase.

It was also verified a negative correlation between SOD and the levels of 3-NT (p= 0,018), which means that when the levels of SOD increase it is observed a decrease in the levels of 3-NT. Conversion of $O_2^{\bullet-}$ to H_2O_2, promoted by SOD, will decrease the levels of $O_2^{\bullet-}$, leading to an exponential decrease in the interaction of NO with $O_2^{\bullet-}$. Thus, production of $ONOO^-$ is consequently decreased. As the levels of this ROS are low, the rate of tyrosine nitration is also affected, occurring a minor production of 3-NT.

5.2. "*APU*" Studies

The "*APU*" study evaluated relationship between OS parameters and the clinical semen parameters. As previously mentioned, ROS promote biomolecules damage in sperm cells, such as lipids, proteins and DNA, which may affect the motility and morphology of sperm cells, among others. To better understand these alterations, spermograms of a total of 32 samples, from a heterogeneous clinical set, were performed. Then, the same parameters were evaluated: the antioxidant capacity, the antioxidant proteins amounts and the protein damage in sperm cells. Our aim was to relate the clinical analysis of sperm cells with their oxidative balance (Table 30).

Table 30 – Overview of the correlations verified in the project "*APU*".

Assay	Relation	Type of correlation	Significance
SOD	Volume	-	0,004
	CG	-	0,005
	Viscosity	-	0,035
	Tail	+	0,044
GPx4	NPM	-	0,012

CG: Carbonyl Groups; GPx4: Glutathione Peroxidase 4; NPM: Non- Progressive Motile Spermatozoa; SOD: Superoxide Dismutase.

In Table 31 are stated the correlations between antioxidant proteins (GPx4 and SOD) and some of the clinical parameters evaluated. SOD was negatively correlated with volume (p = 0,004),

viscosity (p = 0,035) and carbonyl groups (p = 0,005), however it was positively correlated with tail abnormalities of the sperm cells (p = 0,044). In turn, it was also observed a negative correlation between GPx4 and non-progressive motily (p = 0.012).

Glutathione Peroxidase 4 is an antioxidant protein that is mainly present in mitochondria of sperm cells, conferring a high protection to this organelle. In this study, as well as in other previous studies, it was found a negative correlation between motility and the levels of GPx4 (H Imai et al., 2009; H Imai et al., 2001). An increase in the generation of ROS and, consequently, the peroxidation of mitochondrial lipids lead to mitochondria abnormalities that can have negative effects on motility. Glutathione Peroxidase 4 in mitochondria plays a major role in suppressing the effects of ROS, contributing to a better motility of sperm cells. In spermatids, GPx4 is enzymatically active, contributing to deletion of ROS (essentially H_2O_2) preventing the occurrence of mitochondria alterations, such as lipid peroxidation. In mature spermatozoa, GPx4 that is present in mitochondria has a structural role, preventing morphologic alterations, such as tail defects, that can decrease motility. In this study it was found a negative correlation of non-progressive motile sperm cells with the expression of GPx4.

Semen is composed of fluids secreted by the male accessory glands, such as seminal vesicles and prostate. This fluid contains several proteins that are essential to its coagulation and liquefaction. Altered function of prostate and seminal vesicles can lead to hiperviscosity. Oxidative stress is considered to be indirectly a cause of hiperviscosity, due to an increase of leukocytes, which lead to the referred function alteration of the accessory glands cells (Du Plessis, Gokul, & Agarwal, 2013). Oxidative stress is represented by an increase of ROS, but also by a decrease of the antioxidant capacity. Thus, decreased levels of SOD (main antioxidant protein in spermatozoa) can represent the presence of OS that in turn leads to hiperviscosity.

Carbonyl groups assay showed a negative correlation with the presence of SOD (p = 0,005). These results confirmed that SOD protects the spermatozoa against ROS, preventing changes in the side chains of proteins due to the addition of the carbonyl groups (Dalle-Donne, Rossi, Giustarini, Milzani, & Colombo, 2003).

Additionally, some controversial results were also found. Superoxide dismutase was positively correlated with abnormalities in the tail. In this thesis, we analyzed CuZnSOD, an isoform present in the cytoplasm of sperm cells. However, this protein was also observed with a high expression in the testis, more specifically into the spermatogonia of the seminiferous tubules,

as well as into ductus deferens and prostate cells (Nonogaki et al., 1992). An increase of OS in testis is usually accompanied by an increase of OS in the remaining reproductive tract. So, an increase of OS in testis leads to a high expression of CuZnSOD by spermatogonia. However, spermatocytes and spermatids have a low expression of CuZnSOD, being more susceptible to ROS effect in late spermatogenesis, leading to tail defects. When mature spermatozoa with ROS damages are found in epididymis, the seminal fluid donates CuZnSOD to spermatozoa to minimize the effect of ROS in sperm cells. In turn, when OS is observed in testis, which leads to an impairment of spermatogenesis, it is also observed OS in the remaining reproductive tract, and consequently, ductus deferens, seminal vesicles and prostate cells increase their antioxidants production and expression. Consequently, the presence of this protein in seminal fluid increases, being then transported to sperm cells.

At last, it was observed a strong negative correlation between SOD and ejaculate volume. This correlation was also observed by Michałkiewicz et al., however they did not found any information to explain this result (Marzec-Wróblewska et al., 2011). These results are contradictory with the studies developed by Mínguez-Alarcón et al. that refer that an antioxidant intake in diet leads to an increased semen volume (Mínguez-Alarcón et al., 2012).

6. CONCLUSION

In recent years the influence of ROS in many areas of knowledge has been addressed. In sperm cells, ROS play a physiological and a pathological role, depending on the concentration at which they are present. Indeed, ROS are important for capacitation, hyperactivation, acrosome reaction, as well as, for the fusion of spermatozoa with the oocyte. However, when the concentration of ROS increases, the damage of sperm biomolecules occurs leading to a loss of membrane integrity, to DNA fragmentation, and even to apoptosis. ROS levels increase due to the occurrence of an imbalance in the oxidative species (ROS) and antioxidants. This work evaluated this imbalance, analyzing the antioxidant levels, as well as, the consequent protein damage caused by ROS.

The results showed that an acute lifestyle change, specifically the consumption of alcohol and tobacco, was responsible for an alteration in the oxidative balance of sperm cells. Thus, during the first evaluation of the samples after the academic festivities (TP2 – one week later) there was a loss of the antioxidant capacity and consequently an increase in oxidized proteins. However, in a long term period (TP3 – 3 months later), the sperm develop their antioxidant defenses again by increasing the levels of antioxidants, especially CuZnSOD and in turn, the levels of protein oxidation suffered a significant decrease.

After evaluating the importance of alcohol and tobacco consumption alterations in academic week to sperm cells, a comparison between the oxidative balance and the clinical quality of sperm was performed. It was shown that increased levels of antioxidants contribute to a better semen quality, in particular with regard to motility and morphology.

In conclusion, the acute changes in lifestyle during the academic week festivities is responsible for a decreased semen quality, a reduction of sperm normal morphology and motility and by a decrease in the levels of sperm antioxidants. Although the sperm was able to recover from these excesses, this study highlights that these mechanisms are very sensitive to some aggressions and it is possible that in chronic situations (alcoholism and tabagism), these systems could be compromised with a concomitant consequence to fertility.

To follow this study, the antioxidant activity of the studied proteins (SOD and GPx4) can be analyzed after an acute OS stimulus. Then, as mitochondrial GPx4 is inactive in mature sperm cells, expression and activity of other H_2O_2-scavenging proteins, such as CAT, need to be taken into account. Also, it is important to increase the number of samples in future works in order to increase significance.

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8. APPENDIX

8.1. Solutions

SDS-PAGE gels

10% Sodium Dodecyl Sulfate (SDS)

Volume: 10 mL

Compound	Weight	Units
10% SDS	1	g
ddH_2O	10	mL

Store at RT

Stock Solution 30% Acrylamide/0.8% Bisacrylamide

Volume: 500 mL

Compound	Weight	Units
Acrylamide	150	g
Bisacrylamide	4	g

Store at 4ºC protected from light in aluminum foil

Stock Solution 4x Lower Gel Buffer (LGB)

Volume: 250 mL

Compound	Weight/Vol.	Units
1.5 mM Tris-HCl	45.43	g
0.4% SDS	1	g
ddH_2O	187.5	mL

Adjust the pH to 8.8

Bring to the total volume with ddH_2O

Store at 4ºC

Stock Solution 5x Upper Gel Buffer (UGB)

Volume: 250mL

Compound	Weight/Vol.	Units
0.6 mM Tris-HCl	18.17	g
ddH_2O	175	mL

Adjust the pH to 6.8

Bring to the total volume with ddH_2O

Store at 4ºC

10% Ammonium Persulfate (APS)

Volume: 10 mL

Compound	Weight	Units
10% APS	1	g
ddH_2O	10	mL

Store at 4ºC, best if used within 1 week

4x Protein Sample Buffer

Volume: 5 mL

Compound	Weight/Volume	Units
40% Glycerol	2.0	mL
250 mM Tris-HCl pH 6.8	1.3	mL of 1M Tris-HCl pH 6.8
8% SDS	0.4	g
2% β-mercaptoethanol	0.1	mL
ddH_2O	1.65	mL
Bromophenol blue	Add according to the color you want	

Store at RT for short period or 4ºC for longer periods

Can be stored at -20ºC but without adding the β-mercaptoethanol

Western Blot

Stock Solution 10x Running Buffer

Volume: 1000 mL

Compound	Weight	Units
0.25 M Tris-HCl	30.29	g
1.92 M Glycine	144.13	g
1% SDS	10	g

Can be heated to help dissolving the SDS

Adjust the pH to 8.3 and store at RT

Stock Solution 10x Transfer Buffer

Volume: 1000 mL

Compound	Weight	Units
0.25 M Tris-HCl	30.29	g
1.92 M Glycine	144.13	g

The pH should be between 8.2-8.4

Store at RT

Dilute to 1x Transfer Buffer in a volume correspondent to the total volume minus the volume of methanol needed

Add 200 mL of 20% methanol before the transfer

Store at 4ºC if transferring small gels

Stock Solution 10x TBS-T

Volume: 1000 mL

Compound	Weight	Units
0.2 M Tris-HCl	24.23	g
1.5 M NaCl	87.66	g

Bring to the total volume with ddH_2O

Adjust the pH to 7.5

Add 5 mL of 0.5% Tween-20

Adjust the pH to 8.9 and store at 4ºC

5% Blocking Solution

Volume: 100 mL

Compound	Weight	Units
5% Low Fat Milk or BSA	10	g
Add 1x TBS-T	100	mL

Can be stored for a couple of days at 4ºC, best if used fresh

Stripping Solution

Volume: 50 mL

Compound	Weight/Vol.	Units
62 mM Tris-HCl pH 6.7	6.2	mL of 0.5 Tris-HCl pH 6.7 stock solution
2% SDS	1	g
6 mM Urea	18	g

Add 6 mM (18g) of urea per 50 mL of solution

Add 0.7% β-mercaptoethanol before use

8.2. Questionnaire

8.2.1. First Questionnaire

Estudo sobre a influência dos festejos académicos na qualidade do esperma

Questionário Nº1

Nota: Este inquérito é confidencial e destina-se exclusivamente a ser utilizado neste projeto de investigação científica e estudos dele decorrentes. Os dados relativos ao e-mail e contacto telefónico dos participantes serão apenas utilizados para envio dos resultados do espermograma e para prestar informações relacionadas com as fases seguintes do estudo.

Voluntário nº	
E-mail	
Telemóvel (facultativo)	
Data da recolha	
Hora da recolha	
Hora de receção da amostra	
Hora de realização do espermograma	
Pretende receber o resultado do espermograma por e-mail? (Sim/Não)	

Dados gerais

Idade:	
Nº de dias de abstinência sexual:	
Doenças relevantes atuais ou passadas (incluir história de tratamentos oncológicos, parotidite com orquite e consumos atuais de medicamentos ou suplementos alimentares):	
Nº de filhos:	

Consumos habituais

Tabaco (nº médio cigarros/dia no último mês)	
Álcool (média diária do último mês) – descrever o tipo de bebidas e quantidades respetivas	
Drogas (média diária do último mês) – descrever o tipo de drogas e quantidades respetivas	
Drogas (história de consumo no passado)	

8.2.2. Second Questionnaire

Estudo sobre a influência dos festejos académicos na qualidade do esperma

Questionário Nº 2

Nota: Este inquérito é confidencial e destina-se exclusivamente a ser utilizado neste projeto de investigação científica e estudos dele decorrentes. Os dados relativos ao e-mail e contacto telefónico dos participantes serão apenas utilizados para envio dos resultados do espermograma e para prestar informações relacionadas com as fases seguintes do estudo.

Voluntário nº	
E-mail	
Telemóvel (facultativo)	
Data da recolha	
Hora da recolha	
Hora de receção da amostra:	
Hora de realização do espermograma	
Pretende receber o resultado do espermograma por e-mail? (Sim/Não)	

Dados gerais

Idade:	
Nº de dias de abstinência sexual:	
Doenças relevantes atuais ou passadas (incluir história de tratamentos oncológicos, parotidite com orquite e consumos atuais de medicamentos ou suplementos alimentares):	
Nº de filhos:	

Consumos habituais

Tabaco (nº médio cigarros/dia durante a semana académica)	
Álcool (média diária durante a semana académica) – descrever o tipo de bebidas e quantidades respetivas	
Drogas (média diária durante a semana académica) – descrever o tipo de drogas e quantidades respetivas	

8.2.3. Third Questionnaire

Estudo sobre a influência dos festejos académicos na qualidade do esperma

Questionário Nº3

Nota: Este inquérito é confidencial e destina-se exclusivamente a ser utilizado neste projeto de investigação científica e estudos dele decorrentes. Os dados relativos ao e-mail e contacto telefónico dos participantes serão apenas utilizados para envio dos resultados do espermograma e para prestar informações relacionadas com as fases seguintes do estudo.

Voluntário nº	
E-mail	
Telemóvel (facultativo)	
Data da recolha	
Hora da recolha	
Hora de receção da amostra	
Hora de realização do espermograma	
Pretende receber o resultado do espermograma por e-mail? (Sim/Não)	

Dados gerais

Idade:	
Nº de dias de abstinência sexual:	
Doenças relevantes atuais ou passadas (incluir história de tratamentos oncológicos, parotidite com orquite e consumos atuais de medicamentos ou suplementos alimentares):	
Nº de filhos:	

Consumos habituais

Tabaco (nº médio cigarros/dia no último mês)	

Álcool (média diária do último mês) – descrever o tipo de bebidas e quantidades respetivas	
Drogas (média diária do último mês) – descrever o tipo de drogas e quantidades respetivas	
Drogas (história de consumo no passado)	